Influenza-specific CD4+ T_H1 memory cells in the lung can be functionally altered by allergen exposure

Mikel Joseph Ruterbusch

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2023

Reading Committee:

Marion Pepper, Chair

Steven F. Ziegler

Jakob von Moltke

Program Authorized to Offer Degree:

Immunology

University of Washington

Abstract

Influenza-specific CD4+ T_H1 memory cells in the lung can be functionally altered by allergen exposure

Mikel Joseph Ruterbusch

Chair of the Supervisory Committee:

Marion Pepper

Department of Immunology

CD4+ lung-resident memory T cells (T_{RM}) generated in response to influenza infection confer effective protection against subsequent viral exposures. Whether these cells can be altered by environmental antigens and cytokines released during heterologous, antigen-independent immune responses is currently unclear. We therefore investigated how influenza-specific CD4+ T_H1 T_{RM} in the lung are impacted by a subsequent T_H2-inducing respiratory house dust mite (HDM) exposure. Although naïve influenza-specific CD4+ T cells in the secondary lymphoid organs do not respond to HDM, influenza-specific CD4+ T_{RM} in the lungs do respond to a subsequent allergen exposure by altering their phenotype and functional capacity. Changes in transcription factor expression in this population persisted upon heterosubtypic influenza challenge and was associated with decreased morbidity, viral load, and pro-inflammatory cytokine expression in the lung. Further investigation revealed that respiratory cysteine protease or rIL-33 administration was sufficient to induce these changes in the lung-resident influenza-

specific CD4+ T_{RM} population. Thus, heterologous antigen exposure or IL-33 release can drive persistent alterations in CD4+ T_H1 T_{RM} populations.

Table of Contents

List of Figures

Dedication

To my father, Frederick Joseph Ruterbusch. While he was taken far too soon, his exemplary approach to life and the lessons he instilled in me from an early age was an essential source of guidance during the most difficult times of this journey.

Acknowledgements

To my mentor, Dr. Marion Pepper, I cannot overstate the gratitude I possess towards your guidance and encouragement over the many years. Through the ups, downs, diversions, and detours my project has taken, you have continuously taught me how to be a better scientist along the way. Thank you for your patience, long-suffering, and optimism through it all. I will always be indebted to you.

To the entirety of the Pepper lab and its many versions over the last eight years, thank you for being an incredible crew to work with. More than the rigorous and continuous scientific advice, I have shared many laughs and memorable moments with you all that I will always hold on to. I am lucky to call many of you friends as well as colleagues, and I hope to always do so.

Thank you to my thesis committee members, Jakob, Dan, Steve, and Bill, for your invaluable and endless advice and full-fledged support. Your pragmatic approaches to science kept me on track when there were many paths ahead and allowed me to get to this point. I always looked forward to our meetings and hearing your viewpoints throughout the years.

Mrs. Jennifer Traggis and Dr. Jim Miller: I would never have started on this path in life if you did not believe in me. Thank you for taking a chance on me and encouraging me to become a better and more ambitious person.

To my many friends in Seattle and beyond, you have made the tough times easy and the good times exceptional. Thank you to my partner, Alexis, for her unwavering support on this difficult endeavor, even after several years of mentioning I was “probably about six to twelve months” from graduating. And thank you to my Mom and brother, for being the best family members I could ask for, and for supporting me from the very beginning.

1 Introduction

1.1 Initiation of the innate and adaptive immune response

The innate and adaptive immune system are two developmentally distinct yet functionally interconnected networks of immune cells that orchestrate to protect the host organism from harmful pathogens, carcinogenic neoplasms, and promote tissue repair. Loss of critical innate and adaptive immune cell types, or even suppression of specific functions of various immune cells, can upset this balance and lead to severe autoimmune disease or immunodeficiency. The initiation of the immune response occurs primarily upon innate immune cells sensing invariant molecules associated with pathogenic organisms, such as double-stranded RNA and lipopolysaccharide [1-3]. In response, these sentinel immune cells can produce pro-inflammatory type I interferons such as IFN-α and IFN-β that can restrict viral replication and bacterial growth through the induction of interferon-stimulated genes [4-8]. In addition to promoting antiviral programs, type I interferons can also induce expression of costimulatory molecules on innate antigen presenting cells such as dendritic cells (DCs), which is essential in activating the adaptive immune response [9]. The release of inflammatory mediators at the site of infection or antigenic stimulation, such as histamine, leukotrienes, and the cytokines TNF-α and IL-1, can promote vascular permeability and the upregulation of adhesion markers on the vascular endothelium [10]. This promotes extravasation of innate and adaptive immune cells into the tissue and the propagation of the local immune response [11].

Upon antigen exposure or infection, activated migratory and resident antigen presenting cells (APCs) in the draining lymph nodes prime the CD4+ and CD8+ T cell arm of the adaptive immune response [12-14]. In addition to trafficking antigen sampled from the site of infection to the lymph node, soluble antigens which travel through the afferent lymphatics can be captured by lymph node-resident APC populations and presented as peptide:major histocompatibility complex class II (MHC-II) molecules to CD4+ T cells [13-16]. APCs in the lymph node can

activate CD8+ T cells through cross-presentation, a biological phenomenon in which internalized antigens can be presented as peptide:MHC-I molecules, a pathway that is classically restricted to cytosolic-derived antigens [17-19]. The interactions between APCs and CD4+ and CD8+ T cells through peptide:MHC and T cell receptors (TCR), co-stimulatory ligands and receptors, and paracrine cytokine production and sensing initiate and direct the T cell response to a pathogen or antigen. The description of various signals and cellular interactions which orchestrate CD4+ T cell immunity are discussed in further detail in the following section (*1.2 CD4+ T cell activation and differentiation*).

Although the soluble antigens which drain to the local lymph nodes can be sampled and displayed by MHC-I and MHC-II by classical antigen presenting cells, these particles can also stimulate the humoral immune response. Humoral immunity is characterized by the production of antibodies, which can neutralize, opsonize, and activate complement to eliminate pathogens [20]. Antibody producing cells are derived from the B cell lineage and can include plasma cells and plasmablasts found in the secondary lymphoid organs, bone marrow, or non-lymphoid tissue [21, 22]. Aside from antibody production, B cells can also serve important roles in the activation and maintenance of the CD4+ T cell response. Like certain innate immune cell subsets, B cells can express MHC-II on their surface and drive CD4+ T cell activation and differentiation towards a T follicular helper (T_{FH}) cell fate [23, 24]. B cells thus play several pleiotropic roles in the immune system with both adaptive and innate-like qualities, which work in concert to expedite clearance of pathogens at the site of infection through multiple mechanisms.

Overall, various facets of the immune system mediate the elimination of threats that perturb tissue homeostasis by temporally and functionally distinct means. Sensing of invariant molecules present within pathogens or immunogenic antigens can drive rapid antiviral responses, recruitment of a diverse array of immune cells to the site of infection or exposure, and promote adaptive immune cell activation. The sampling, processing, and presentation of

foreign antigens in the local lymph node can further stimulate T and B cell responses that are essential for clearance of many pathogens in mice and humans. Although many of these components of the immune system are indispensable for protection in various contexts, the focus of this dissertation is on the role of CD4+ T cells in respiratory viral infection.

1.2 CD4+ T cell activation and differentiation

CD4+ T cell subset differentiation: an overview

From a T cell-centric perspective, pathogens and various environmental antigens can be classified by the type of immune response they elicit. CD4+ T cells can commit to distinct programs comprised of unique cytokine profiles that dictate the features of the resulting immune response, theoretically in a targeted fashion to optimally eliminate the pathogen or stimulus [25-27]. These classically include the T_H1 program, which is characterized by IFN-γ and TNF-α production to eliminate viruses and intracellular bacteria, T_H2 differentiation defined by secretion of IL-5 and IL-13 in response to allergens or helminths, and T_H17 cells that generate IL-17 to combat fungal infections [28]. The commitment to these programs can occur during the initial activation of these cells, termed the priming phase, and in some cases be reinforced as these cells encounter further immune signals at the site of infection or antigen exposure [28, 29]. The induction and expression of master transcription factors which control the expression of these subset-associated genes are necessary for lineage commitment in CD4+ T cells [28]. Seminal work from multiple groups approximately a quarter century ago identified T-bet as the master transcription factor for T_H1 cells, GATA-3 for T_H2 cells, and RORγT for T_H17 cells [28, 30-32]. The tipping point in an immune response that can lead to either effective or defective pathogen clearance or the avoidance or development of immunopathology or autoimmunity can many times be attributed to cues that influence effector subset commitment.

In addition to the various effector subsets that CD4+ T cells can commit to upon activation, it has more recently been appreciated that these cells can differ by their activation state as well [28]. While still incorporating cues and expressing cytokine profiles that are consistent with $T_H1/T_H2/T_H17$ differentiation, CD4+ T cells can concomitantly establish their fate as effector T cells (T_{eff}) that produce high levels of cytokines or T follicular helper cells that support and direct B cell responses [28]. Like the T cell subsets, transcription factors dictate and demarcate these activation states, with Blimp-1 driving T_{eff} cell differentiation and Bcl-6 leading to T_{FH} formation [28, 33-36]. Yet the factors and signals that influence these fate decisions in various contexts continue to be defined in recent and ongoing work, demonstrating that CD4+ T cells can exhibit remarkable heterogeneity [28].

T cell receptor signaling: more than just an activation checkpoint

The first event that occurs when a naive CD4+ T cell is activated is the interaction of the TCR with a cognate peptide:MHC complex on an antigen presenting cell. However, in addition to controlling activation of the T cell, TCR signaling can also impact differentiation. One of the first examples of this was described by Paul and colleagues [37]. Using TCR transgenic mice, they discovered that lower concentrations of peptide induced early phosphorylation of STAT5 and production of IL-4 and GATA-3 by CD4+ T cells *in vitro* [37]. Conversely, high peptide concentrations abolished early GATA-3 and IL-4 production, which could be reversed by inhibiting MEK [37]. Their work demonstrated that antigen concentration may affect T cell differentiation and responsiveness to various cytokines. This observation was later recapitulated *in vivo*, where antigen concentration was found to dictate CD4+ T cell polarization over the type of adjuvant used [38]. Here, high levels of antigen led to increased IL-12R expression and T_H1 differentiation [38]. Thus, antigen load and therefore TCR signal strength can affect cytokine receptor expression and CD4+ T cell polarization.

In addition to altering effector subset polarization, TCR signal strength can also mediate the activation state of CD4+ T cells. Tubo et al. addressed this phenomenon by performing an experiment where congenically marked naive CD4+ T cells were transferred into hosts such that the donor cells would contain on average one naive CD4+ T cell specific for LLO, a *Listeria monocytogenes*-derived peptide [39]. Following infection with *Listeria*, the CD4+ T cells specific for LLO were purified and their differentiation and activation state was assessed. They found that each naive CD4+ T cell clone has a distinct cell lineage or differentiation potential that is dependent on TCR signal strength [39]. Cells that experienced a short duration of TCR signaling became T_{FH} cells, whereas T_{eff} were induced by a longer interaction with peptide:MHC-II [39]. Of note, cells experiencing the longest interaction and thus TCR signaling became germinal center T_{FH}, suggesting that TCR signal strength can influence CD4+ T cell activation state commitment along a continuum [39]. This finding is supported by another recent study, which demonstrated naïve CD4+ T cells that received the highest TCR signals were induced to produce IL-2 and become committed to the T_{FH} lineage, with lower TCR signaling linked to IL-2 non-producers and T_{eff} differentiation [40]. However, some studies using *in vivo* models suggest stronger TCR stimulation is associated with T_{eff} differentiation, suggesting possible differences depending on context or subset definition [41, 42].

In an *in vivo* vaccination setting, high and low stability peptides used to prime CD4+ T cells were able to generate T_H1 cells that produce IFN-γ upon infection with influenza [43]. However, only low stability peptides could induce a population of CD4+ T cells that have the capacity to produce IL-17A following infection [43]. The T_H1 cells generated by high-stability peptides proliferated poorly following infection, suggesting that increased TCR signal strength may generate terminally differentiated effector cell subsets that are suboptimal for vaccination strategies [43]. Together, the current body of evidence delineates the necessity for further study of how TCR signal strength can influence CD4+ T cell differentiation, with considerations pertaining to the infection model employed and nomenclature used to define various CD4+ T

cell subsets and activation states [38]. However, it is clear that TCR signal strength during the initial steps of CD4+ T cell activation can govern both functional outcome and polarization by impacting transcription factor and cytokine receptor levels that can affect responsiveness to the local milieu and guide cell fate before other differentiating signals come into play.

Interleukin-2: a gas pedal and steering wheel that drives CD4+ T cells to their destination

Activation of naive CD4+ T cells ultimately requires not only TCR recognition of peptide:MHC-II, but also perception of IL-2 and signaling through the IL-2 receptor (IL-2R). The balance of these events with other differentiating signals controls cell fate and polarization. Downstream of the IL-2R is STAT5 (among other pathways), which becomes phosphorylated and forms homodimers or heterodimers that translocate into the nucleus and control gene transcription. Johnston et al. found that STAT5-deficient CD4+ T cells rapidly express Bcl-6 and preferentially form T_{FH} cells following LCMV infection [44]. Conversely, CD4+ T cells that constitutively express an active form of STAT5 become T_{eff} cells and fail to form T_{FH} or germinal centers [44]. This is in line with previously discussed work which demonstrated CD4+ T cells that are not IL-2 producers themselves yet perceive paracrine IL-2 preferentially become T_{eff} [40]. To further support these findings, Randall and colleagues showed that IL-2 administration impaired T_{FH} cell formation and limited the generation of germinal cells during influenza infection [45]. Similar findings on IL-2R signaling and STAT5 activation on early CD4+ T cell activation and fate has been uncovered in humans, suggesting that this is a conserved and consistent feature of CD4+ T cell immunity [46]. Thus, increased signaling through the IL-2R can chaperone CD4+ T cells towards a T_{eff} lineage through STAT5 phosphorylation, indicating that IL-2 plays an essential role in CD4+ T cell activation state commitment.

In addition to regulating the activation state of CD4+ T cells, the presence and strength of STAT5 signaling can also impact polarization. STAT5-deficient CD4+ T cells exhibit severely attenuated T_H2 responses both *in vitro* and *in vivo* [47, 48]. IL-2 acts through STAT5 to mediate

accessibility of the IL-4 gene, and IL-2 blockade or the absence of STAT5 results in reduced responsiveness to IL-4 receptor signaling and a loss of IL-4 production [48]. The concentration of antigen present can also affect STAT5 signaling, as low antigen levels have been shown to lead to early STAT5 phosphorylation and IL-4 and GATA-3 production in CD4+ T cells [37]. As a whole, STAT5 phosphorylation driven through IL-2R signaling is able to control effector subset differentiation of CD4+ T cells in addition to guiding their activation state.

Antigen presenting cell subsets that provide differentiating signals

In addition to TCR signal strength and perception of soluble factors, the identity of the antigen presenting cell that activates a CD4+ T cell can also dictate its differentiation and activation state. Using histocytometry, Gerner et al. found that draining antigen forms a concentration gradient in the draining lymph node, with higher amounts of antigen located near the periphery [16]. The cDC2s that can present peptide:MHC-II and activate CD4+ T cells are located near the periphery and therefore require lower antigen doses to prime and activate CD4+ T cells in comparison to cDC1s that are located in the deep T cell zone and activate CD8+ T cells [16]. Following antigen administration, T_{FH}-promoting DCIR2+ CD4+ DCs that express ICOSL and localize to the outer T cell zone to interact with EBI2+ CD4+ T cells can promote a T_{FH} cell fate [49]. These DCs also produce soluble and membrane bound forms of CD25 to quench local IL-2 to further reinforce T_{FH} differentiation [49]. Migratory DCs can also prime T_{FH} cells, as CD11b+ cDC2s were found to express homing molecules that position them at the T:B border upon entry into the lymph node following intranasal OVA+LPS administration [50]. Mice that are deficient in cDC2s have a reduction in protective antibody generation following infection with inactivated influenza virus, demonstrating their importance in generating T_{FH} cells that can provide B cell help [50]. Although antigen presenting cells play a significant role in driving cell fate during the activation of naive CD4+ T cells, they can also impact cell function in non-lymphoid tissue during later stages of infection.

During local antigen exposure in the skin, effector CD4+ T cells can interact with antigen presenting cells, cease migration in a transient fashion, produce a burst of cytokine, and then continue to migrate [51]. This is dependent on PD-1/PD-L1 interaction, as blockage of PD-L1 on antigen presenting cell leads to longer dwell time of the CD4+ T cells and greater amounts of cytokine production [51]. This dependence of cytokine production on interaction with antigen presenting cells and TCR signaling was further demonstrated *in vivo* in the settings of *Mycobacterium tuberculosis* and influenza infection [52]. In these contexts, cytokine production by CD4+ T cells in the lung is restricted to areas of high antigen abundance, and thus influenza infection generates more cytokine production in the lung than *M. tuberculosis* due to higher antigen load [52]. Thus, succeeding the differentiating signals that are imparted on CD4+ T cells at the site and time of priming by APCs and the cytokine milieu, local antigen presence and presentation in non-lymphoid tissue can further control the levels of cytokine produced by CD4+ T cells.

1.3 Formation and maintenance of CD4+ T cell memory

Formation of CD4+ memory T cells

Following disappearance of the immune stimulus, whether an acute infection or antigen exposure, the number of immune cells drastically subsides in comparison to those found shortly after the generation of the immune response [53]. Although many of the cells found during the acute phase fail to persist, subsets of CD4+ T cells, termed memory CD4+ T cells, remain poised to rapidly and efficiently respond upon antigen reencounter [53]. As with CD4+ T cell effector subsets and activation states, CD4+ T cell memory cells are compartmentalized into distinct fates that differ in their phenotype, function, and localization [53, 54].

One of the first demonstrations of distinct CD4+ memory T cell subsets was performed by Lanzavecchia and colleagues in human peripheral blood [54]. Here they identified the effector memory (T_{EM}) subset, which lacks surface expression of the chemokine receptor CCR7

and retains markers for tissue migration, and central memory (T_{CM}) cells which are CCR7+ and home to the lymphoid tissue [54]. In addition to expressing distinct phenotypic markers and localization tendencies during the memory phase, T_{EM} and T_{CM} also give rise to various CD4+ T cell subsets following reactivation. Upon cognate antigen recognition, T_{EM} primarily become effector T cells, while T_{CM} are able to differentiate into both T_{FH} and effector T cells [53-55]. Thus, altering the balance of memory subsets that are formed following infection or antigen exposure could have functional consequences upon reactivation through the generation of effector T cells or T_{FH}.

More recently, another memory T cell subset which is distinct from T_{EM} and T_{CM} has been described in both mice and humans. Tissue resident memory T cells (T_{RM}) are almost exclusively found in the non-lymphoid tissue at the site of infection or antigen exposure, but unlike T_{EM}, do not recirculate under homeostatic conditions [56-60]. Although work from Masopust and others has demonstrated that T_{RM} can potentially seed a pool of memory cells that are resident in the draining lymph node, the majority of CD4+ T_{RM} patrol the local tissue and remain primed to respond upon a secondary antigen exposure [60-63]. These CD4+ T_{RM} stationed in the tissue can play indispensable and critical roles in providing protection against re-infection (reviewed in section 1.5), or in the case of allergy, can promote immunopathology [56, 58-60]. While T_{EM} and T_{CM} are still important components of an intact and effective immune recall response in many scenarios, T_{RM} are essential mediators of tissue-restricted immunity.

Similar to how various cell intrinsic and extrinsic signals can drive differentiation of effector CD4+ T cells, several groups have delineated the factors that promote CD4+ memory T cell formation. In a polyclonal setting, each CD4+ T cell clone is capable of producing memory CD4+ T cell progeny, suggesting that memory cell formation is not restricted to certain TCR clonotypes [64]. Memory T cells can arise from effector CD4+ T cell progenitors, although this can be dependent on the perception of various signals during specific phases of the immune response [65-69]. A critical component of CD4+ memory T cell generation is sensing of IL-2 [59,

66, 70]. IL-2R signaling during contraction of the acute immune response directs effector CD4+ T cells to become memory cells [66]. Further demonstrating the role of IL-2 in memory formation, mice that lack high affinity IL-2R on their CD4+ T cells possess a severely limited number of T_{RM} at the site of infection or allergen exposure [59, 70]. In addition to IL-2 driving CD4+ memory T cell formation, the expression of the transcription factor Thpok can promote the differentiation and fitness of memory cells by antagonizing Blimp-1 and Runx3 expression [71]. Thus, the formation of memory CD4+ T cells *in vivo* can be regulated by the temporal sensing of environmental cues by effector CD4+ T cells. There remains some debate, however, whether memory T cell differentiation strictly occurs in a linear fashion, or if memory potential can be differentially imprinted on daughter cells during initial T cell activation [72].

Maintenance of CD4+ memory T cells

During an acute immune response, T cell clones proliferate in response to TCR signaling and sensing of pro-survival cytokines such as IL-2. However, once the infection or inflammatory stimulus subsides, CD4+ memory T cells must persist in the absence of cognate antigen and a cytokine miliieu which favors cell survival. Upon T cell sensing of IL-2 and transition to a memory fate, CD4+ memory T cells upregulate IL-7R which is essential for their maintenance [66, 73-75]. IL-7R signaling drives expression of Bcl-2, an anti-apoptotic transcription factor which mediates the persistence of CD4+ memory T cells in the absence of antigen [74]. IL-7 deficient mice or intact mice which experienced a temporal absence of IL-7R signaling via antibody blockade failed to maintain CD4+ memory T cell populations [74]. In addition to IL-7, the cytokine IL-15 also plays important roles in CD4+ memory T cell maintenance [73, 76]. In multiple contexts, IL-15 has been demonstrated to regulate homeostatic proliferation of CD4+ memory T cells once formed, although the initial differentiation of memory cell populations appears to be more reliant on IL-7 [73, 76]. Together, these cytokines play essential roles in the generation and/or maintenance of CD4+ memory T cells *in vivo*.

Although IL-7R and IL-15R signaling are essential contributors to CD4+ memory T cell survival, a number of structural and cellular factors can also regulate the maintenance of memory cells. In the genital mucosa, macrophages can produce chemokines that support protective CD4+ T_{RM} generated in response to herpes simplex virus 2 infection [77]. In LCMV infection, B cell deficient mice have increased numbers of antigen-specific CD4+ T cells in the lungs during acute infection, but their persistence during the memory phase is severely impacted [70]. B cells and T cells have been identified in close proximity in a number of tissues during memory or late timepoints in mice and humans [59, 78-81]. The localization of these cells within the tissue is typically within loosely-organized lymphoid clusters which resemble T cell zones and B cell follicles present in secondary lymphoid organs. These clusters are typically positioned in adventitial cuffs near larger structures of the vasculature and airways and are termed inducible bronchus associated lymphoid tissue (iBALT) [78, 79, 81-83]. Induction of iBALT is dependent on IL-17, but independent of antigen-specific immunity [78, 79]. iBALT formation can be induced by LPS or other inflammatory stimuli and form high endothelial venules and these structurues are a location of naïve T cell activation or memory CD4+ T cell maintenance [78, 79, 81]. Taken together, the formation of specialized immune structures in non-lymphoid tissue can retain CD4+ memory T cells and be a source of cellular or soluble factors that promote the maintenance of memory T cell populations.

1.4 Lung-lived memory: CD4+ resident memory T cells in respiratory viral infection

Following the resolution of the acute immune response at the site of infection, the non-lymphoid tissue retains a pool of memory lymphocytes. These include B cells as well as CD8+ and CD4+ T cells which remain resident in the tissue as sentinel cells in the context of re-infection or antigen re-exposure [60, 84, 85]. In contrast to circulating memory cells, these tissue-resident memory lymphocytes have been described to possess unique roles in protection

upon re-infection or in driving enhanced immunopathology in the setting of allergic disease [58, 59].

One particular study that demonstrated the protective capacity of tissue-homing memory lymphocytes in humans was performed following the 2009 H1N1 influenza pandemic [86]. Here, the investigators analyzed immune correlates of protection in patients that did not have pre-exisiting neutralizing antibodies but possessed cross-reactive cellular immunity [86]. They identified an IFN-γ-expressing CCR7- CCR5+ CD8+ T cell population with lung-trafficking potential that correlated with less severe disease upon H1N1 infection, suggesting these tissue-homing CD8+ T cells can protect against heterosubtypic infection [86]. Another study analyzed nasal swabs from antibody-naïve individuals following influenza infection and found that CD4+ T cells reactive to H1N1 peptide pools correlated with improved viral clearance and showed evidence of cytotoxic activity [87]. These studies demonstrate that both CD4+ and CD8+ memory T cells with the capacity to home to or directly located in the respiratory tissue play an essential role in providing protection against re-infection, even in the absence of neutralizing antibody.

Although these human studies rigorously defined correlates of protection, seminal work from Farber and colleagues used mouse models to more directly interrogate the antiviral capacity of CD4+ memory T cells in the tissue. Using TCR transgenic mice which possessed CD4+ T cells specific for influenza hemagglutinin, the investigators demonstrated that influenza-specific tissue-resident memory CD4+ T cells formed in the lung following infection are sufficient to drive decreased morbidity and viral load following re-infection [58]. Notably, this tissue-resident population provides enhanced protection in comparison to influenza-specific memory CD4+ T cells isolated from the spleen in the same mice [58]. These influenza-specific memory CD4+ T cells also possess the ability to reduce the viral burden in infected mice in the absence of endogenous lymphocytes, suggesting that at least a portion of their function in this context is boosting innate immunity in myeloid or stromal cells or perhaps direct lysis of infected cells,

although this was not directly investigated [88-92]. These studies demonstrate that CD4+ T_{RM} are distinct in comparison to other memory CD4+ T cell subsets in both their localization and function and are essential mediators of antiviral protection provided by the immune system.

Even though CD4+ memory T cells in the tissue can provide protection against re-infection in the absence of endogenous lymphocytes, a major role of tissue-localized CD4+ memory T cells during infection is to assist local CD8+ T cell and B cell responses [89, 93-97]. Recently, two separate groups have described a subset of T_{FH}-like Bcl-6+ $FR4^{hi}$ $PD\text{-}1^{hi}$ resident CD4+ memory T cells (T_{RH}) in the lung that promote local CD8+ T cell populations and antibody production in influenza infection through secretion of IL-21 [93, 94]. As has been described in CD4+ T_{RM} populations in other infections or inflammatory contexts, these cells localize to iBALT and are dependent on B cells for their maintenance in the lung [93, 94]. In the absence of CD4+ T cells, the formation, maintenance, localization, and recall of CD8+ memory T cells in the lung is severely impacted and leads to impaired viral clearance [95, 96]. More specifically, the production of IFN-γ by CD4+ memory T cells in the lung drives proper CD8+ T cell differentiation in T_{RM} cells and mediates their antiviral function upon re-infection [95]. Similar mechanisms of CD4+ T cell-mediated protection were observed in SARS-CoV-1 infection, suggesting that these findings are not restricted to influenza infection alone [97]. Thus, CD4+ memory T cells localized to the tissue or airway respiratory viral infection are essential mediators of a robust antiviral response, either through direct lysis of infected cells, or the activation or orchestration of the innate or adaptive immune cells.

1.5 Aeroallergens and immunity

The first step in the immune response to allergens occurs during their interaction with the epithelium at the site of exposure. In response to sensing pathogen associated molecular patterns such as LPS, or experiencing structural damage from protease activity, epithelial cells produce pro-inflammatory and damage-associated cytokines such as IL-33, TSLP, IL-25, and

TNF-α [104]. These signals from the epithelium are the first line of defense and set in motion a signaling cascade that can result in a robust T_H2 immune response at the site of exposure.

One of the primary molecular initiators of the T_H2 immune response in barrier tissues is TSLP [105, 106]. TSLP production has been linked to PAR2 signaling in keratinocytes and the induction of cutaneous itch sensations [107, 108]. Once produced, TSLP can act on multiple cell types to promote T_H2 responses in a context-dependent manner, including dendritic cells, ILC2s, basophils, and CD4+ T cells [105, 106]. In both *in vitro* and *in vivo* settings, TSLP has been demonstrated to induce IL-13 production in CD4+ T cells and promote T_H2 terminal differentiation in the tissue [29, 105, 106, 109]. Specifically, TSLPR signaling works in a synergistic fashion with IL-4R signaling to generate pathogenic CD4+ T_H2 cells that produce high levels of IL-5 and IL-13 in humans and mice [110]. Thus, TSLP plays a critical role in promoting T_H2 inflammation through a wide array of cell types, even as a cytokine that is induced early in the immune cascade.

Another driver of T_H2 immunity that can be initiated at the level of the epithelium is IL-25. The cells that primarily express IL-25 in the gut epithelium are tuft cells, and although tuft cells can be identified in the respiratory mucosa, they are typically restricted to the nasal cavity or trachea [111-113]. However, IL-25 still plays a role in the distal lung, as blockade of IL-25 in mice attenuates the T_H2 responses in the lung tissue [114]. IL-25R, composed of IL-17RA/IL-17RB heterodimer, is expressed by several immune cells, including CD4+ T cells, ILC2s, and innate effector cells [29, 112]. The administration of IL-25 in the respiratory tract leads to enhanced T_H2 cytokines in the lung and generation of IgE antibodies, demonstrating its function as a potent driver of type 2 immunity [115]. The importance of tuft cells and IL-25 in driving T_H2 immune responses and other biological phenomena is a rapidly evolving field, although it is well-appreciated that IL-25 can play unique roles in multiple contexts and tissues [112].

The function of IL-33 in promoting a T_H2 response is well-studied, and pleiotropic roles for IL-33 continue to be discovered. Although allergens and various pathogens stimulate

upregulation of IL-33 by epithelial cells, it must also be released into an environment where it can interact with immune cells. IL-33 contains no signal peptide to allow for its secretion and is targeted to the nucleus and binds chromatin. Thus, its release is thought to be limited to mechanical necrosis or programmed pro-inflammatory cell death [116]. Apoptotic cells inactivate full-length IL-33 through caspase-3 and 7 processing [117]. However, a broad array of allergens can also directly process full-length IL-33 in the extracellular environment into a form that more potently induces T_H2 cytokine production, eosinophilia, and mucus production [118]. The tightly controlled regulation of IL-33 to be released and active only under circumstances of cell injury or destruction suggests that IL-33 and the downstream T_H2 response is a critical component of wound repair [116]. The hijacking of this response by allergens cleaving full-length IL-33 into a highly immunogenic form demonstrates how these seemingly innocuous antigens can induce a robust immune response and chronic disease.

Epithelial sensing of pathogen associated molecular patterns, such as LPS and flagellin, can initiate a cascade that promotes T_H2 immunity. In the absence of TLR signaling in epithelial cells using a cell-specific knockout of MyD88, eosinophilia in response to house dust mite or administration of OVA and flagellin was virtually absent [98]. MyD88 is also involved in the signaling cascade of the IL-33 receptor, however, which was not ruled out as a potential pathway that could lead to this observation in this study. A meta-analysis found that polymorphisms in TLR2 and TLR4 are associated with an increased risk of developing allergic asthma in humans, demonstrating the role of these early signaling pathways in the development of allergic disease [99]. However, allergens are not inert molecules that facilitate an immune response strictly due to TLR agonists that are present in the source material. The house dust mite-derived allergen Der p 2 possesses structural homology to MD-2, a TLR4 co-receptor, and can enhance signaling of TLR4 in the presence of LPS [100]. In addition to functional mimicry, allergen proteases can also cleave tight junction proteins to facilitate entry into the tissue [101]. Mite proteases have also been shown to cleave several cell surface receptors that are present

on immune cells [102, 103]. Thus, allergens can stimulate a broad cascade of signals that drive T_H2 immune responses, and consequently, hallmarks of allergic disease.

APC subsets that propagate the allergic response

Following initiation of the immune response at the epithelium, these signals are relayed to the adaptive immune system by antigen-presenting cells and other innate cells. Various subsets of these cells have been demonstrated to be critical for the initiation of the T_H2 response. In a house dust mite-induced asthma model, migratory CD11b+ conventional DCs, which can first sense cytokines and alarmin signals at the site of exposure, were shown to be major drivers of T_H2 initiation [119]. During allergen challenge in the lung, monocyte-derived DCs were shown to be sources of cytokines that supported T_H2 cells already present in the tissue, and largely dispensable for T_H2 cell initiation [119]. Proteases present in allergens such as cockroach feces can activate DCs and amplify their T_H2 cytokine production and allergic airway inflammation in a PAR2-dependent manner [120]. Migratory DCs isolated from the lung can imprint homing characteristics to CD4+ T cells by inducing CCR4 expression and drive them to the lung under both homeostatic and inflammatory conditions at a greater frequency than DCs isolated from other sites [121]. Lung-resident alveolar macrophages also likely play a role in the development of asthma, as replacement of these cells by monocyte-derived alveolar macrophages following murid herpesvirus-4 infection led to a reduction in the T_H2 response if these mice were later exposed to house dust mite (HDM) [122].

In the skin and gut, CD301b+ DCs were shown to be important for transporting antigen to the draining lymph node and eliciting a T_H2 response in response to alum or papain exposure or *Nippostrongylus brasiliensis* infection [123]. Recent work in a cutaneous allergy model using papain demonstrated that CD301b+ DCs require both CCR7 and CCR8 to enter into the lymph node parenchyma and drive T_H2 differentiation [124]. The ligand for CCR8, CCL8, was determined to be derived from CD169+ SIGN-R1+ F4/80+ macrophages located in the

interfollicular regions of the lymph node following papain treatment [124]. The exact stimulus that initiates CCL8 production by these macrophages is currently unclear. Our current understanding of T_H2 response, where these CD11b+ and CD301b+ migratory dendritic cell subsets are essential for T_H2 cell differentiation, demonstrates that the spatial gap between the site of allergen exposure and T_H2 cell initiation is bridged by these migratory DCs that can relay cytokine signals and antigen to the site of T cell priming. However, once primed in the lymph node, T_H2 cells can themselves sense signals in non-lymphoid tissue to become terminally differentiated and exhibit full effector function [29]. One of the important sources of these signals is a macrophage subset that has been demonstrated to localize to the bronchi and serve as an APC in the tissue which promotes local effector T_H2 responses to allergen [160].

In addition to dendritic cell and macrophage subsets, the induction of the T_H2 response following allergen exposure in the lung is facilitated in part by ILC2s [125]. This reliance of the T_H2 response on ILC2s is circumvented when allergen or adjuvants are administered during the priming phase by intraperitoneal injection [125]. This further demonstrates the involvement of cells at the site of exposure relaying primary signals from the epithelium to augment downstream T_H2 responses. In fact, ILC2 activation in the lung following intranasal papain administration was shown to be IL-33-dependent [126]. This IL-33-dependent activation of ILC2s induced expression of IL-13 which promoted dendritic cell migration to the lymph node and T_H2 differentiation [126]. During the memory phase, ILC2-derived IL-13 upon antigen re-exposure in the lung or skin can be directly sensed by DCs and generate production of CCL17, the ligand for CCR4 [127]. This enhances recruitment of memory T_H2 cells that express CCR4 such as CD4+ T_{EM} [127]. It is unclear if this ILC2/IL-13/DC axis is necessary or sufficient for the reactivation of CD4+ T_H2 T_{RM}, but it nevertheless appears to affect the magnitude of the T_H2 response by recruiting circulating memory T cells.

The role and functional capabilities of ILC2s is still an emerging field, but their ability to augment epithelial cell-derived signals and dictate antigen-presenting cells to initiate and

sustain T_H2 responses is well-supported. ILC2s may also be able to present antigen, as MHC-II expression is observed on ILC2s and they exhibit the ability to endocytose and process antigen [128]. Adoptive transfer of MHC-II-deficient ILC2s into ILC2-deficient mice led to impaired clearance of *Nippostrongylus brasiliensis* compared to mice that received wild-type ILC2s [128]. Thus, ILC2s likely interact with T_H2 cells and play a direct role in their function in addition to ILC2s orchestrating DC migration and polarization.

1.6 Outstanding questions

While the signals that drive the formation and differentiation of CD4+ T_{RM} in non-lymphoid tissue have been elucidated to some degree, whether these cells can be altered once established has yet to be studied. It is currently accepted that once a CD4+ T_{RM} cell has differentiated and become entrenched in the tissue, it is resistant to changing its expression of lineage defining transcription factors and its cytokine profile in the absence of further engagement through the TCR. Thus, any perturbations to the tissue or cytokine milieu through subsequent infections or environmental antigen exposures would not alter the fate or function of previously formed CD4+ T_{RM}. However, this has not directly been investigated. Any plasticity of CD4+ T_{RM} populations in response to secondary immune stimuli has broad and significant implications for vaccine or therapeutic design and is therefore an area of importance. For example, allergen exposure following vaccination or natural infection could alter the phenotype or protective capacity of previously generated CD4+ T_{RM} and make the assessment of these populations acutely after their formation inaccurate. Additionally, therapeutic strategies could be developed to target previously formed CD4+ T_{RM} to boost or modulate their function in contexts where their current state is insufficient to provide protection upon re-infection or has the potential to drive immunopathology.

In the studies presented here, we use tetramer-based approaches to demonstrate that CD4+ T_{RM} formed in response to influenza infection can alter their master transcription factor

expression, surface receptor expression, and cytokine profile following respiratory allergen exposure. We defined that these changes in this influenza-specific CD4+ T_{RM} population following allergen exposure persist upon re-infection and are associated with decreased morbidity, viral load, and pro-inflammatory cytokine production in the lung. In chapter 4, we assessed the pathways that govern these alterations in influenza-specific CD4+ T_{RM} after HDM exposure and found that rIL-33 or papain, but not IL-18 or LPS, could drive similar phenotypic changes in these cells. These studies define that CD4+ T_H1 T_{RM} formed in the lung tissue can respond to the surrounding immune environment and alter their phenotype and function independent of antigen.

2.1 Materials and methods

Mice

Female C57BL/6J mice were purchased from The Jackson Laboratory and maintained under specific pathogen free conditions at the University of Washington. Red5 (B6(C)-Il5$^{tm1.1(icre)Lky}$/J) and Great (C.129S4(B6)-Ifng$^{tm3.1Lky}$/J) mice were also purchased from The Jackson Laboratory and maintained under specific pathogen free conditions at the University of Washington. Mice that were infected with influenza A virus Puerto Rico/8/34 H1N1 (PR8) or influenza A virus HKx31 H3N2 (X31) were housed in Animal Biosafety Level-2 conditions. Experiments were performed in accordance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Viral infections

Mice were anaesthetized with ketamine/xylazine and intranasally instilled with 10 plaque forming units (pfu) of influenza A virus PR8 or 1000 pfu units of influenza A virus X31 diluted in sterile PBS in a final volume of 40 μL. For experiments that utilized FTY720 (Fingolomod; Enzo

Life Sciences), mice were injected intraperitoneally daily for the indicated range with 25 μg FTY720 in sterile diH20.

Immunizations

For preparation of HDM immunizations, whole crushed *D. pteronyssinus* house dust mite powder (Greer Laboratories Inc., Lenoir, NC) was re-suspended in sterile PBS. For experiments that performed allergic sensitization and challenge, mice were anaesthetized with isofluorane and administered HDM via the oropharynx at a concentration containing 23 μg Der p 1 protein in a volume of 40 μL during the primary sensitization. Beginning at 10 days-post sensitization, mice were anaesthetized with isofluorane and instilled with HDM in the oropharynx at a concentration containing 5.75 μg Der p 1 protein daily for five days during the allergic challenge phase.

For acute respiratory challenge experiments with HDM, papain, LPS, and recombinant cytokines, mice were anaesthetized with isofluorane and administered the target molecules in a total volume of 40 μL diluted in sterile PBS via the oropharynx for the indicated days. On each day, mice were given HDM normalized to 5.75 μg Der p 1 protein (Greer Laboratories Inc.), 25 μg papain (from papaya latex, aseptically filled, Sigma-Aldrich), 10 μg Ultrapure LPS from E. coli O111:B4 (InvivoGen, San Diego, CA), 50 ng recombinant mouse IL-33 (Biolegend), and/or 500 ng recombinant mouse IL-18 (Biolegend) as described in the relevant figures.

Isolation of cells from the lung

Approximately 3 minutes prior to sacrifice, mice were injected intravenously with 1 μg anti-Thy1.2 BUV395 (clone 53-2.1, BD Biosciences) to label T cells in the vasculature [129]. Mice were then euthanized via CO2 asphyxiation and lungs were harvested into PBS with 2% fetal

calf serum. The lung tissue was placed into gentleMACS C Tubes (Miltenyi Biotec) with RPMI 1640 Medium with HEPES (Gibco, #22400089) containing 70 μg/mL Liberase TM (Roche #05401127001) and 10 mM aminoguanidine (Sigma-Aldrich). The tissue was dissociated on the gentleMACS Dissociator (Miltenyi Biotec) and then incubated at 37°C for 30 minutes followed by a final dissociation step. The single cell suspensions from the lung were then filtered over 70 μm mesh and washed with Dulbecco's Modified Eagle's Medium [Corning, Inc.; Ref: 10-017-CV] with 10% fetal calf serum to inhibit liberase activity.

Quantitative Reverse Transcription PCR

For viral RNA quantification, the right middle lung lobe was harvested from C57BL/6J mice and placed in RNAlater Stabilization Solution (Invitrogen) at -20°C. 15 mg of lung tissue was lysed using the RNeasy Plus Mini Kit RLT buffer with β-mercaptoethanol (Qiagen) and a 5mm stainless steel bead (Qiagen) on the TissueLyser II (Qiagen) twice for 2.3 mins at 30 Hz. RNA was isolated from the lysate using the RNeasy Plus Mini Kit (Qiagen) according to manufacturer instructions. Isolated RNA was then synthesized and amplified into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher). For IAV M1/M2 measurement, this cDNA was used as a template for quantitative PCR using PrimeTime qPCR Probe Assays (Integrated DNA Technologies, Inc.) and PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Inc.) with the following primer and probe design: Probe: (6-FAM/ZEN/IBFQ) 5'- CCTCTGCTGCTTGCTCACTCGATC-3'; Forward Primer: 5'-CAGCACTACAGCTAAGGCTATG-3'; Reverse Primer: 5'-CTCATCGCTTGCACCATTTG-3'. Transcripts were normalized to Rps17 (40S ribosomal protein S17) which was quantified using PowerUP SYBR Green Master Mix (Applied Biosystems) with the primers: *Rps17* Fwd: 5'-CGCCATTATCCCCAGCAAG-3'; *Rps17* Rev: 5'- TGTCGGGATCCACCTCAATG-3'. For IL-6, IFN-γ, and TNF-α quantification, the following primers were used with PowerUP SYBR Green

Master Mix and normalized to *Rps17*. *IL6* Fwd: 5'- TGAACAACGATGATGCACTTG-3'; *IL6* Rev: 5'- CTGAAGGACTCTGGCTTTGTC-3'; IFNG Fwd: 5'-ATGAACGCTACACACTGCATC-3'; IFNG Rev: 5'-CCATCCTTTTGCCAGTTCCTC-3'; *TNF* Fwd: 5'- TCTGTCTACTGAACTTCGGGGTG-3'; *TNF* Rev: 5'- ACTTGGTGGTTTGCTACGACG-3'. Quantitation was performed on the ViiA7 Real-Time PCR System (Applied Biosystems).

In vitro stimulation and intracellular cytokine staining

After acquiring a single cell suspension from lung tissue digestion (see Isolation of cells from the lung), cells were pelleted and re-suspended in 25 ng/mL PMA and 1.4 μM Ionomycin in Dulbecco's Modified Eagle's Medium [Corning, Inc.; Ref: 10-017-CV] with 10% fetal calf serum for 4.5 hours at 37°C. For the final 3.5 hours of culture, BD GolgiStop (BD Biosciences) containing Monensin was added according to manufacturer instructions. Following incubation, the cells were filtered over nitex mesh (amazon.com) and stained with APC-conjugated $NP_{311-325}$:I-A^b tetramer for 1 hour at room temperature. Cells were then washed and stained for surface markers (CD4, clone: GK1.5, BD Biosciences; B220, clone: RA3-6B2, BD Biosciences; LIVE/DEAD Fixable Blue Dead Cell Stain Kit, Invitrogen; Purified CD16/CD32, clone 2.4G2, BD Biosciences) for 30 minutes on ice. Cells were then fixed and permeabilized with eBioscience Foxp3/Transcription Factor Staining Buffer Set (Invitrogen) according to manufacturer instructions. Intracellular cytokine staining (IFN-γ, clone: XMG1.2, Biolegend; IL-10, clone JES5-16E3, Biolegend; IL-13, clone eBio13A, Invitrogen) was performed in 1X Permeabilization Buffer (Invitrogen) at room temperature for 1 hour before flow cytometry acquisition.

Cell enrichment and flow cytometry

Following acquisition of single cell suspensions, cells were stained with $NP_{311-325}$ (QVYSLIRPNENPAHK) I-A^b tetramer or Der p $1_{117-127}$ (CQIYPPNVNKI) I-A^b as indicated

conjugated to APC or PE and incubated at room temperature in the dark for 1 hour. Cells were then washed and incubated with 25 μL anti-APC and/or anti-PE microbeads (Miltenyi Biotec) on ice for 30 minutes. After incubation, cells were washed and tetramer-positive cells were enriched over magnetic LS columns (Miltenyi Biotec) as previously described [130, 131]. The enriched fraction and "flow-through" non-enriched fraction were then surfaced stained with identical antibody master mixes on ice for 30 minutes for downstream analysis of tetramer-specific cells and bulk lymphocyte populations. When applicable, cells were then fixed and permeabilized with eBioscience Foxp3/Transcription Factor Staining Buffer Set (Invitrogen) according to manufacturer instructions. Intracellular transcription factor staining was performed in 1X Permeabilization Buffer (Invitrogen) at room temperature for 1 hour before data acquisition by flow cytometry. All cells were acquired on the LSR II or FACSymphony (BD) and analyzed using FlowJo 10.8.1 software (Treestar). All plots shown in figures are pre-gated on FSCxSSC singlet B220- CD8- CD4+ cells. Naïve CD4+ cells as indicated are further gated on CD62L+ CD44- CD69- cells.

For surface staining, the following antibodies were used: B220 (clone: RA3-6B2; BD), CD4 (clone: RM4-5 or GK1.5; Biolegend or BD), CD8 (clone: 53-6.7; BD), CD3 (clone: 145-2C11; BD), CD44 (clone: IM7; BD), CD69 (clone: H1.2F3; BD), CD62L (clone: MEL-14; BD), CD218a (clone: P3TUNYA; Invitrogen), ST2 (clone: DJ8; MD Bioproducts), Thy1.2 (clone: 53-2.1; BD). For intracellular transcription factor staining, the following antibodies were used: T-bet (clone: 4B10; Invitrogen), GATA3 (clone: L50-823; BD), Foxp3 (clone: FJK-16s; Invitrogen).

Statistical analysis

Statistical analysis was performed by unpaired t test, Mann-Whitney test, or Spearman's rank correlation as indicated in the figure legends using Prism 9.4.0 (GraphPad Software, San Diego, CA). Graphs show mean ± SD.

3 Phenotypic and functional modulation of influenza-specific CD4+ resident memory T cells following respiratory house dust mite exposure

3.1 Introduction

Influenza poses a significant global health burden through seasonal epidemics and the threat of emergent pandemic strains. Lung-resident memory CD4+ T cells generated in response to influenza can provide robust protection upon re-infection [58, 89]. CD4+ T cells are critical regulators of both B cell and CD8+ T cell responses yet can also provide protection in the absence of CD8+ T cells and B cells [93-96]. Once these cells are formed, it is unclear how subsequent heterologous pathogen or environmental antigen exposures impact this population or alter its functionality.

Prior studies demonstrated that the in vivo reactivation of memory CD4+ T_H1 cells in secondary lymphoid organs is restricted by CD4+ T cell cognate recognition of peptide:MHC complexes [132]. Whether or not memory cells in the tissues are similarly restricted or how different inflammatory conditions may alter a memory T cell response was not examined [132]. Interestingly, work by Paul and colleagues demonstrated that CD4+ T_H2 memory cells in the lung could exacerbate subsequent T_H2 immune responses to HDM in a TCR-independent manner through IL-33 [133]. Following sensing of IL-33, these CD4+ T_H2 memory cells produced the effector cytokines IL-5 and IL-13 in the absence of TCR signaling and promoted increased eosinophilia and worsened pathology [133]. Another IL-1 family member cytokine, IL-18, has also been implicated in prompting similar functional enhancement of CD4+ T_H1 responses [134, 135]. Upon sensing of IL-18, CD4+ T_H1 cells can produce IFN-γ independently of TCR stimulation when paired with STAT4 induction in vitro [134]. Although these studies have investigated how existing T_H1 or T_H2 CD4+ T cells can be further enhanced by similar environmental cues, it remains unclear how an existing T_H1 T_{RM} population can be impacted by a subsequent T_H2 stimuli such as common environmental allergens. We therefore sought to

define how influenza-specific CD4+ T_H1 T_{RM} cells in the lung responds to a subsequent allergen exposure (**Fig. 3.1**).

To answer this question, we developed a model system in which we could study two non-cross-reactive CD4+ T cell populations with different functional outputs: a predominantly T_H1-skewed memory population specific for the influenza nucleoprotein (NP; $NP_{311-325}$:I-Ab) and a T_H2-skewed population specific for the common allergen Der p 1, a protease produced by the house dust mite *Dermatophagoides pteronyssinus* (Der p $1_{117-127}$:I-Ab) [59, 136, 137] (**Fig 3.1**). These tools allowed us to first demonstrate a lack of activation or cross-reactivity between these two naïve epitope-specific populations, as naïve NP-specific cells do not proliferate or differentiate in response to HDM exposure, nor do naïve Der p 1-specific cells respond to influenza. We next investigated whether tissue-resident NP-specific T_H1 memory cells generated in response to influenza A/Puerto Rico/8/34 H1N1 (IAV PR8) infection could respond to an irrelevant T_H2-inducing HDM exposure. While we did not see an overall expansion of NP-specific CD4+ T_{RM} cells in response to HDM, we did find that these cells were phenotypically and functionally impacted by the subsequent allergen exposure. Specifically, a diminished proportion of NP-specific CD4+ T_{RM} retained expression of their lineage-defining transcription factor (T-bet), a change which persists for at least 25 days post-HDM exposure. These cells also exhibit altered functionality following allergen exposure as they produce less IFN-γ upon stimulation than NP-specific CD4+ T cells isolated from HDM unchallenged, IAV-exposed mice. Of interest, allergen-exposed influenza-specific CD4+ T cells also increased expression of the IL-33R receptor (ST2), yet did not become GATA-3+ T_H2 cells, demonstrating limited plasticity [138]. As expression of IL-33R can be regulated by IL-33 perception, we further tested whether administration of papain or rIL-33 could similarly impact the NP-specific T_H1 population [139]. We observed that respiratory administration of papain or rIL-33 was sufficient to drive a lower frequency of T-bet expression in NP-specific CD4+ memory cells in the lung, demonstrating that induction of IL-33 independent of cognate antigen can alter previously established CD4+ T_H1

T_{RM}. Together, these data provide novel insights into how environmental antigens can alter the fate and function of viral-specific CD4+ T_{RM} in the lung, suggesting new strategies for therapeutic interventions.

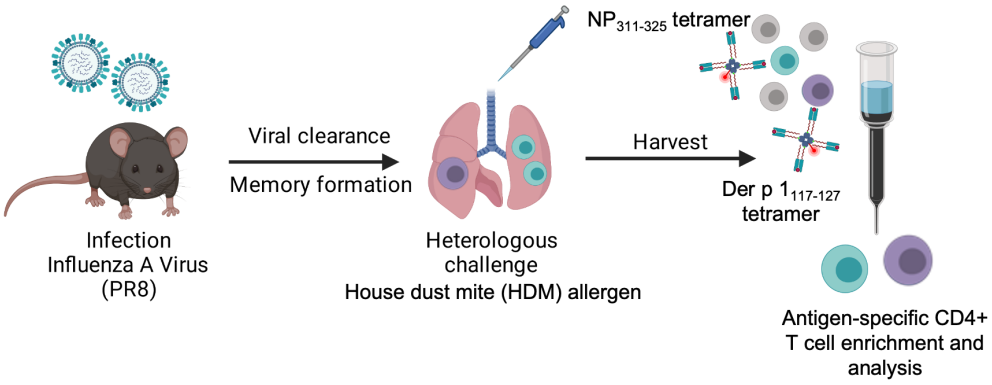

Figure 3.1. Experimental overview.

A schematic illustrating the overarching experimental approach taken to address the effect of heterologous antigen challenge on the phenotype and function of resting influenza-specific CD4+ memory T cell populations in the lung.

3.2 Results

3.2.1 $NP_{311-325}$:I-Ab-specific and Der p $1_{117-127}$:I-Ab-specific CD4+ T cells are not cross-reactive or activated by heterologous responses

We developed a system to examine how a lung-resident CD4+ T_H1 T_{RM} population can be impacted by subsequent exposure to an irrelevant, common environmental allergen. Magnetic bead enrichment of dual tetramer-stained CD4+ T cells combined with intravascular labeling prior to sacrifice was performed to identify $NP_{311-325}$:I-Ab and Der p $1_{117-127}$:I-Ab tetramer-specific CD4+ T cells in the lung-draining mediastinal lymph node (dLN) and lung parenchyma at various timepoints following infection and/or allergen administration [130]. To determine the extent of overlapping responsiveness or cross-reactivity, NP-specific and Der p 1-specific CD4+ T cells were identified at timepoints of peak CD4+ T cell expansion in mice after

induction of allergic airway inflammation with HDM (day 18; **Fig. 3.2A**, top) or after infection with IAV PR8 (day 8; **Fig. 3.2A**, bottom) [59]. In mice that were exposed to HDM, a significant Der p 1-specific, but not NP-specific, CD4+ T cell population was present in the dLN and lungs four days after the final HDM dose (**Fig. 3.2A, 3.2C**). Conversely, at an acute time point after IAV PR8 infection, NP-specific but not Der p 1-specific CD4+ T cells expanded in both tissues compared to cell numbers in naïve mice (**Fig. 3.2A, 3.2C**). At neither acute time point were any Der p 1- and NP-specific double positive CD4+ T cells identified, demonstrating that these two epitope-specific populations are not only non-responsive to the other antigenic insult, but were also not directly cross-reactive (**Fig. 3.2A**) [140].

Although we did not observe cross-reactive responsiveness in a heterologous priming environment, CD4+ T_{RM} cells may be more promiscuous and gain responsiveness to a heterologous challenge in an antigen-independent manner [133, 141]. We therefore quantified NP-specific CD4+ T cells in the dLN and lungs at a memory timepoint after IAV PR8 infection alone or at the same time point (day 43) in mice that were additionally challenged with HDM (**Fig. 3.2B**). There was no significant difference in the number of NP-specific CD4+ T cells identified in either the dLN or lungs of mice that did or did not undergo a subsequent HDM challenge, demonstrating that allergic sensitization and challenge is not sufficient to alter the size of the influenza-induced NP-specific CD4+ T_{RM} population once established (**Fig. 3.2B, 3.2C**). Overall, these data demonstrate that IAV PR8 infection induces a population of NP-specific memory cells in the dLN and lungs that are not numerically enhanced by subsequent HDM exposure.

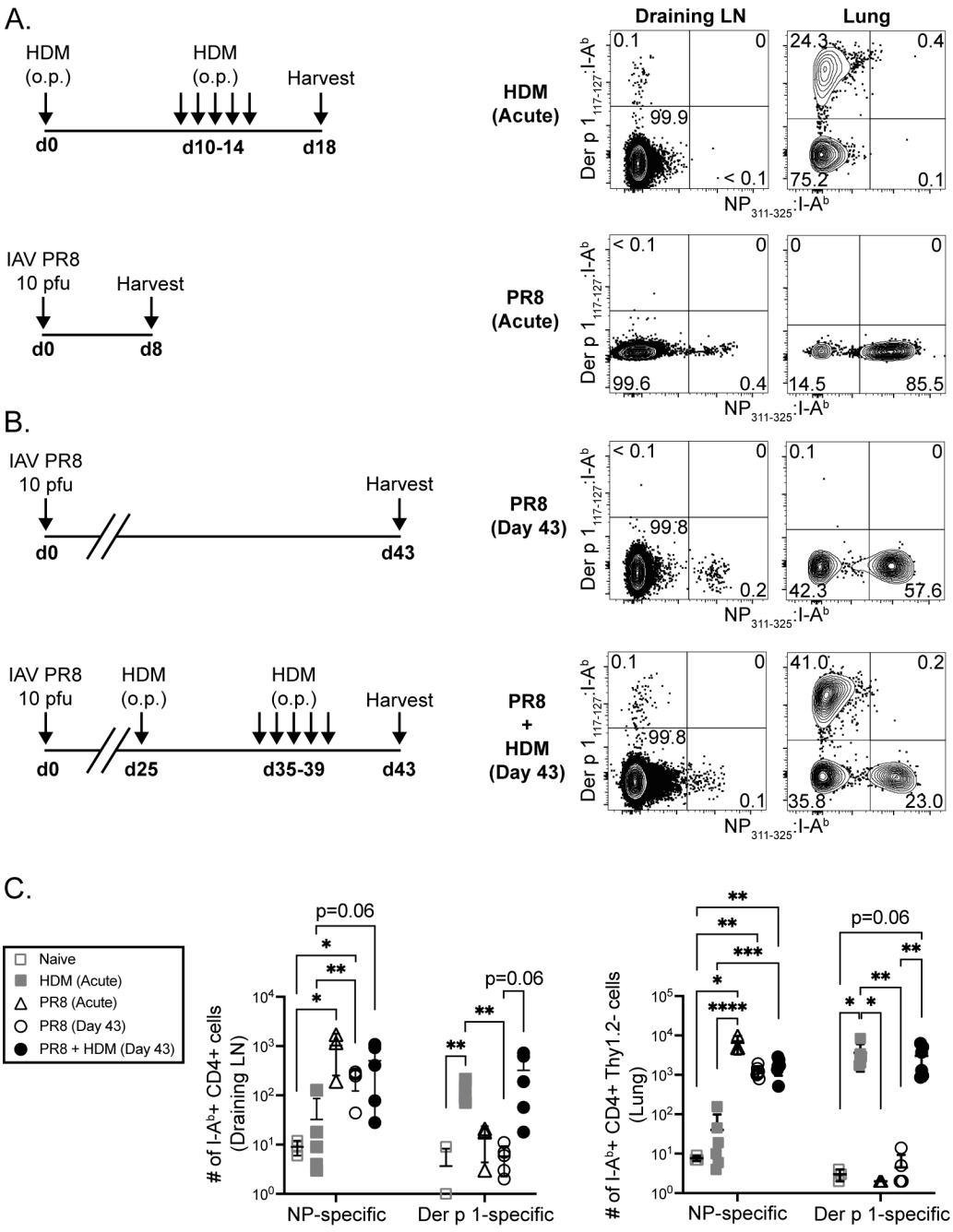

Figure 3.2. $NP_{311-325}$:I-A^b-specific and Der p $1_{117-127}$:I-A^b-specific CD4+ T cells are not cross-reactive or activated by heterologous responses.

(A) In mice administered respiratory HDM (top) or IAV PR8 (bottom) as illustrated in the experimental timeline shown, lung-draining mediastinal lymph nodes and lung tissue were isolated and stained with Der p $1_{117-127}$:I-A^b and IAV $NP_{311-325}$:I-A^b tetramers and analyzed by

flow cytometry. Representative flow plots are shown. Lung samples were pre-gated on cells that are negative for the intravenous Thy1.2 label to demarcate cells located in the lung parenchyma. **(B)** Representative flow cytometry plots of CD4+ T cells isolated from the mediastinal lymph node or lung 43 days post-infection with IAV PR8 (top) or following IAV PR8 and respiratory HDM (bottom) as illustrated in the experimental timeline shown. Lung samples were pre-gated on cells negative for the intravenous Thy1.2 label to demarcate cells located in the lung parenchyma. **(C)** Summary data of the total number of NP-specific or Der p 1-specific tetramer-positive cells in the mediastinal lymph node or lungs of mice from each condition with mean ± SD. Data are pooled from 3-6 mice per group from three independent experiments and were analyzed by unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

3.2.2 HDM-induced airway inflammation following IAV PR8 infection decreases the frequency of T-bet-expressing NP-specific CD4+ T_{RM} in the lung

While it was clear the NP-specific cells were not expanding in response to HDM in the dLN or lungs at either acute or memory timepoints, it was formally possible that an altered environment associated with airway inflammation could impact functional attributes of the NP-specific memory CD4+ T cells. To test this possibility, we compared hallmark characteristics of NP-specific CD4+ cells from the lungs of mice infected with IAV PR8 alone to those additionally exposed to HDM (**Fig. 3.3A**). Specifically, we analyzed the expression of the lineage-defining transcription factor T-bet as influenza induces a strong T_H1 response, characterized by the expression of the T-bet-regulated cytokine IFN-γ [30]. Prior studies have also suggested that IL-1 cytokine family members can enhance CD4+ T cell function in an antigen-independent manner, thus we also examined expression of the IL-33R component ST2, which can be expressed by both T_H1 and T_H2 CD4+ T cells [142, 143]. After IAV PR8 infection, the majority of NP-specific CD4+ T_{RM} cells in the lung express higher amounts of T-bet than naïve antigen non-specific CD4+ T cells as expected (**Fig. 3.3B, 3.3C**). Yet within this T-bet+ population we also found significant heterogeneity with respect to IL-33R expression at memory timepoints after infection; some cells expressed T-bet and but not ST2 (T-bet SP), while others expressed both T-bet and ST2 (DP). After HDM challenge, we observed a lower frequency and level of T-bet expression in the lung-resident NP-specific CD4+ T cell population compared to mice exposed to influenza infection alone, suggesting that HDM exposure can diminish T-bet expression in this NP-specific CD4+ T_{RM} population (**Fig. 3.3B, 3.3C**). Furthermore, the frequency of NP-specific CD4+ T cells that express ST2 increased after HDM challenge, with this increase occurring primarily in T-bet- (ST2 SP) and not T-bet+ (DP) cells (**Fig. 3.3B-D**). Thus, although sensitization and heterologous challenge with HDM is not sufficient to induce expansion of NP-specific CD4+ T cells in the lung, it is able to modulate the expression of a key

transcription factor and cytokine receptor in this population, suggesting subsequent exposure to T_H2 stimuli can impact immune memory previously formed in response to viral infection.

Exposure to a common environmental allergen resulted in a lower frequency of NP-specific CD4+ T cells that expressed the T_H1 lineage-defining transcription factor T-bet and also exhibited enhanced ST2 expression. This finding suggested the possibility that allergic airway inflammation was potentially skewing these T_H1 memory cells towards a T_H2 lineage. CD4+ T_H2 cells express high amounts of ST2 and the T_H2-lineage defining transcription factor GATA-3, yet low amounts of T-bet [29, 144-145]. We therefore repeated the same IAV PR8 infection in the presence or absence of allergic airway inflammation to include an examination of the expression of GATA-3 in the NP-specific CD4+ T_{RM} population. Following challenge with HDM, NP-specific CD4+ T_{RM} in the lung did not express GATA-3 at a higher frequency or level than in unchallenged memory mice (~5%) or form a bona fide GATA-3+ ST2+ population, suggesting HDM exposure does not direct these cells towards a T_H2 fate (**Fig. 3.3E, 3.3F**). As we did not observe the NP-specific CD4+ T_{RM} skew towards a T_H2-phenotype, we next assessed if secondary allergen exposure directed these cells towards a regulatory T cell lineage. In mice that were exposed to HDM following IAV PR8 infection, we did not observe a change in the frequency or level of Foxp3 expression in the NP-specific CD4+ T_{RM} population in the lung, suggesting that allergic challenge also does not cause these cells to become regulatory T cells (**Fig. 3.3G, 3.3H**). Taken together, these data suggest HDM exposure following IAV PR8 infection impacts the frequency of T-bet and ST2 expression in the NP-specific CD4+ T_{RM} population in the lung but does not drive these cells towards a T_H2 or regulatory CD4+ T cell fate.

Although we observed phenotypic and functional alterations in the NP-specific CD4+ T cell population in the lung after HDM exposure, it was unclear whether these changes were a transient response to acute allergic challenge or a longer-term change. Therefore, we next examined if a lower frequency of T-bet expression in NP-specific CD4+ T_{RM} following allergen

exposure was maintained for several weeks after the conclusion of allergic challenge. We performed the same protocol of IAV PR8 infection and allergic airway induction but waited to examine the NP-specific cells until 25 days after the final allergic challenge to determine if the decreased frequency of T-bet+ NP-specific CD4+ T_{RM} was maintained (**Fig. 3.3I**). We observed that mice which received a subsequent allergic airway challenge maintained a lower frequency of T-bet+ NP-specific CD4+ T_{RM} in the lung than those that were only infected with IAV PR8, suggesting the impact of allergen exposure on the CD4+ T_{RM} population persists after the conclusion of challenge with HDM (**Fig. 3.3J, 3.3K**). Of note, the overall frequency of ST2+ NP-specific T_{RM} was not altered in mice that underwent allergic challenge at this later timepoint, suggesting the increased expression of ST2 on NP-specific CD4+ T_{RM} soon after allergic challenge shown in Fig. 2.2 may be transient (**Fig. 3.3J, 3.3K**). However, the increased frequency of ST2-expressing T-bet- (ST2 SP) cells in mice that were sensitized and challenged with HDM was maintained, albeit less dramatically, suggesting that allergen exposure can drive some NP-specific T_{RM} to persistently express ST2 in the T-bet- population at a higher frequency than in mice infected alone (**Fig. 3.3J, 3.3L**). Taken together, these data suggest the decrease in T-bet and increase in ST2 on T-bet- NP-specific CD4+ T_{RM} that arise after allergic challenge is not transient and persists at least up to 25 days after allergic challenge.

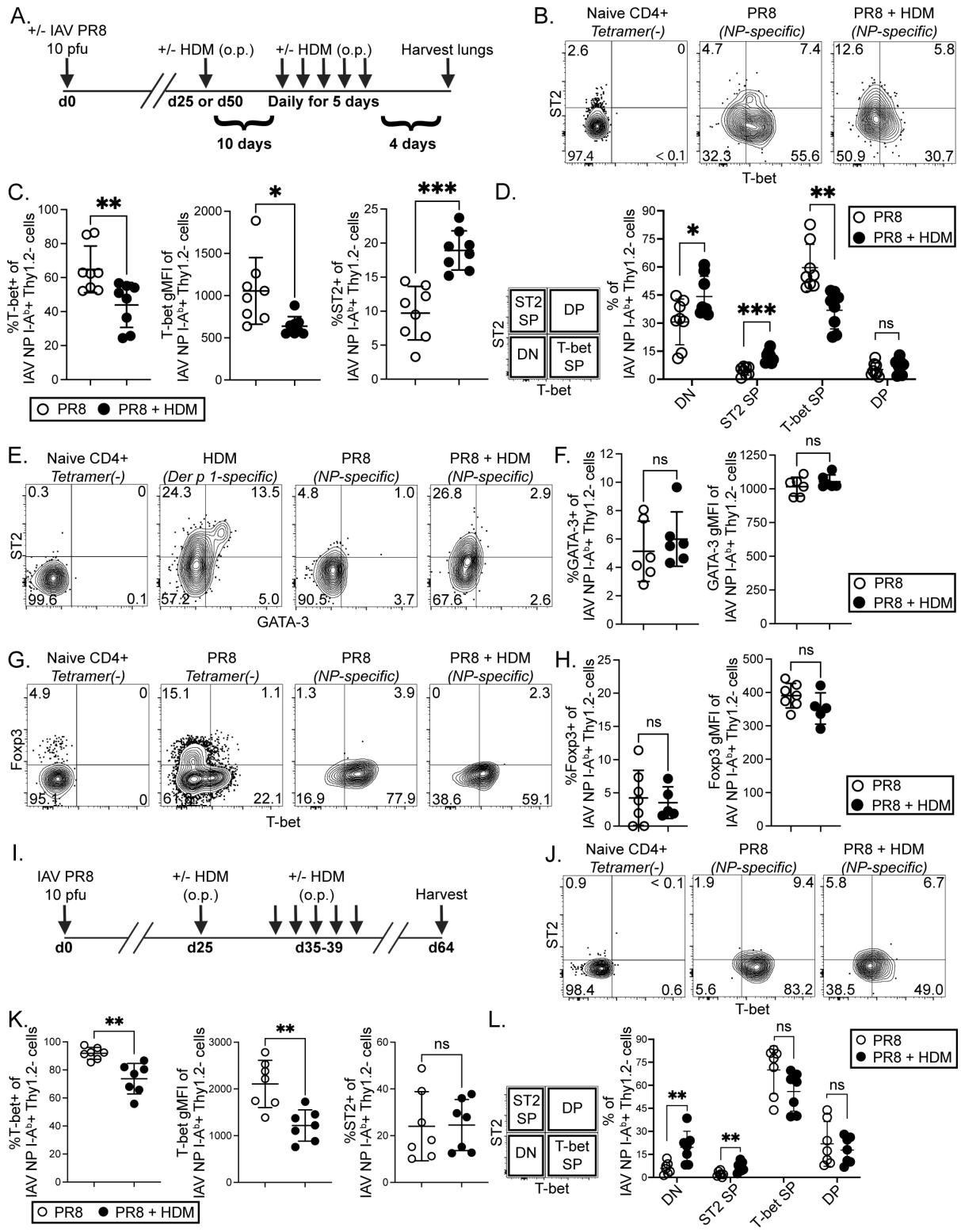

Figure 3.3. HDM-induced airway inflammation following IAV PR8 infection decreases the frequency of T-bet-expressing NP-specific CD4+ T_{RM} in the lung.

(A) Schematic representing the experimental timeline. **(B)** Representative flow cytometry plots of CD4+ T cells isolated from the lung at the timepoint indicated in A. **(C)** Summary data of the percentage of T-bet+ NP-specific cells, mean fluorescence intensity of T-bet in NP-specific cells, and the percentage of ST2+ NP-specific cells as represented in B. Graphs show mean ± SD. Data are pooled from 8 mice per group from two independent experiments and were analyzed by unpaired *t* test. **(D)** Summary data of the percentage of NP-specific cells located within each quadrant based on ST2 and T-bet expression as represented in B. Graphs show mean ± SD. Data are pooled from 8 mice per group from two independent experiments and were analyzed by unpaired *t* test. **(E)** Representative flow cytometry plots of CD4+ T cells isolated from the lung at the timepoint indicated in A. **(F)** Summary data of the percentage of GATA-3+ NP-specific cells and mean fluorescence intensity of GATA-3 in NP-specific cells as represented in E. Graph shows mean ± SD. Data are pooled from 6 mice per group from two independent experiments and were analyzed by unpaired *t* test. **(G)** Representative flow cytometry plots of CD4+ T cells isolated from the lung at the timepoint indicated in A. **(H)** Summary data of the percentage of Foxp3+ NP-specific cells and mean fluorescence intensity of Foxp3 in NP-specific cells as represented in G. Graph shows mean ± SD. Data are pooled from 5-7 mice per group from two independent experiments and were analyzed by unpaired *t* test. **(I)** Schematic of the experimental timeline for data in J-L. **(J)** Representative flow cytometry plots of CD4+ T cells isolated from the lung at experimental day 64. **(K)** Summary data of the percentage of T-bet+ NP-specific cells, the mean fluorescence intensity of T-bet in NP-specific cells, and the percentage of ST2+ NP-specific cells as represented in J. Graphs show mean ± SD. Data are pooled from 7 mice per group from three independent experiments and were analyzed by unpaired *t* test. **(L)** Summary data of the percentage of NP-specific cells located within each quadrant based on ST2 and T-bet expression as represented in J. Graphs show mean ± SD. Data are pooled from 7 mice per group from three independent experiments and were analyzed by unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. DN,

double negative. ST2 SP, ST2 single positive. T-bet SP, T-bet single positive. DP, double positive.

3.2.3 NP-specific CD4+ T_{RM} in the lung exhibit a decreased potential to produce IFN-γ following allergic sensitization and challenge

IL-33 induced by allergen exposure can drive effector cytokine production in CD4+ T_H2 memory T cells in the lung [133]. As we observed NP-specific CD4+ T_H1 T_{RM} responded to allergic challenge by altering their phenotype, we next assessed if HDM exposure induced production of T_H1 or T_H2 effector cytokines in these cells. We performed IAV PR8 infection followed by HDM exposure in mice heterozygous for both IL-5 tdTomato (B6(C)-*Il5*$^{tm1.1(icre)Lky}$/J) and IFN-γ YFP (C.129S4(B6)-*Ifng*$^{tm3.1Lky}$/J) reporter alleles and isolated cells from the lung one day following conclusion of allergic challenge (**Fig. 3.4A**). Approximately half of the NP-specific CD4+ T cells isolated from the lung of mice infected with IAV PR8 expressed IFN-γ at day 40 post-infection, but IL-5 expression was absent (**Fig. 3.4B, 3.4C**). In mice challenged with HDM after IAV PR8 infection, the frequency of IFN-γ or IL-5 production in NP-specific CD4+ T_{RM} remained unchanged (**Fig. 3.4B, 3.4C**). This data suggests that respiratory exposure to HDM following influenza infection does not induce effector cytokine expression in NP-specific CD4+ T_H1 T_{RM} in the lung.

T-bet-driven production of IFN-γ by CD4+ T cells mediates protection to heterosubtypic influenza challenge [30, 89]. As our data demonstrated a loss of T-bet expression by NP-specific CD4+ T cells in the context of allergic airway inflammation, we next sought to determine if the cytokine profile of these cells was altered following HDM exposure. We therefore used the same protocol of IAV PR8 infection followed by allergic airway induction with HDM, but this time isolated NP-specific CD4+ memory T cells from the lung and stimulated them with a protein kinase C activator (PMA) and calcium ionophore (Ionomycin) (**Fig. 3.4D**). While about 50% of NP-specific CD4+ T_{RM} from mice infected with IAV PR8 possessed the capacity to produce IFN-γ upon restimulation, NP-specific cells isolated from the lung after induction of airway inflammation produced approximately two-fold less IFN-γ (~28%) at the same timepoint post-

infection (**Fig. 3.4E, 3.4F**). Consistent with our transcription factor analysis in Fig. 3.3, we observed no change in the capacity of NP-specific CD4+ T_{RM} to produce IL-13, and a small, but significant decrease in IL-10 production potential (**Fig. 3.4G, 3.4H**). Therefore, decreased T-bet expression in NP-specific CD4+ T cells upon allergen exposure is also associated functionally with a decreased frequency and level of IFN-γ and IL-10, but not IL-13 expression.

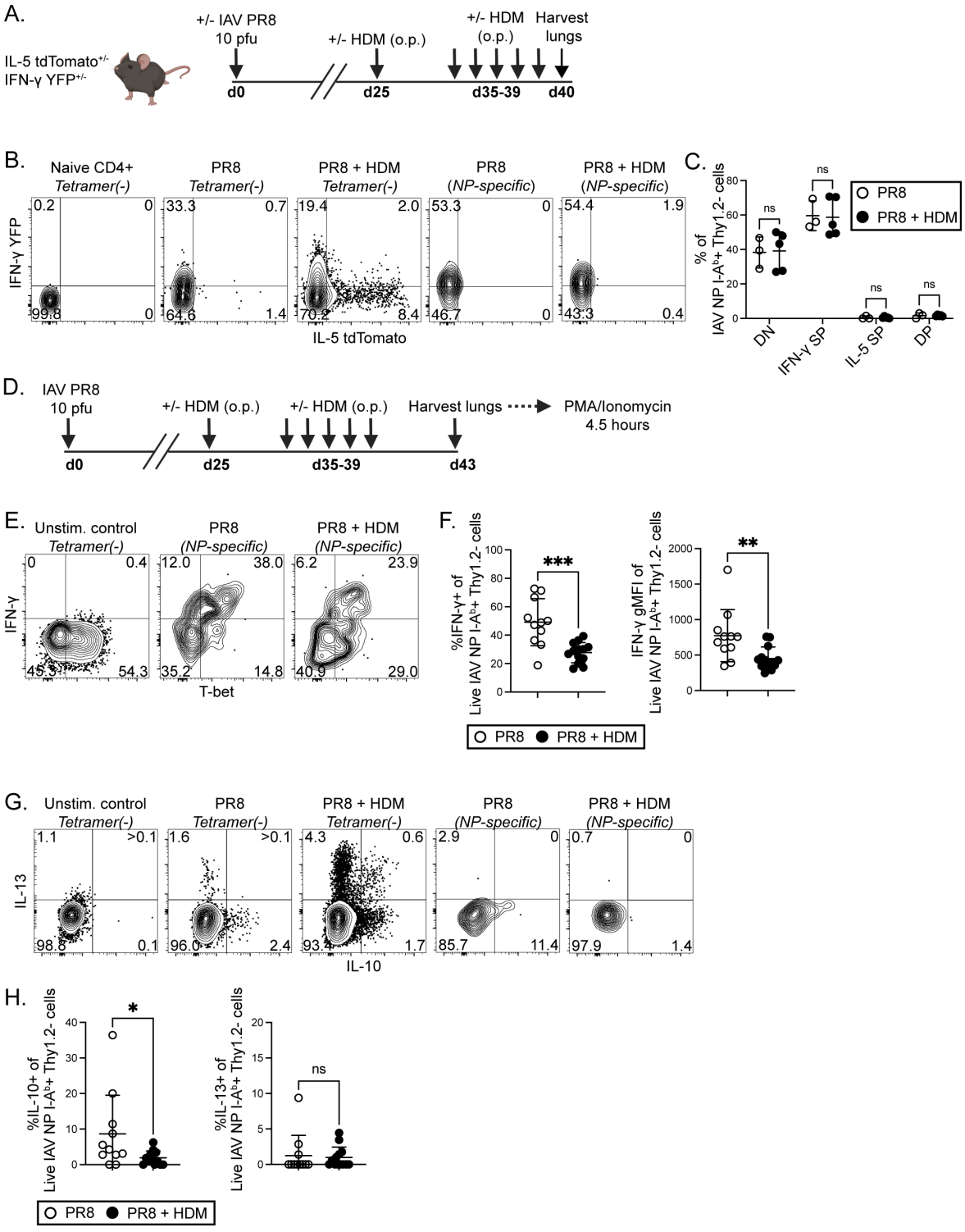

Figure 3.4. NP-specific CD4+ T_{RM} in the lung exhibit a decreased potential to produce IFN-γ following allergic sensitization and challenge.

(A) Schematic of the experimental timeline. **(B)** Representative flow cytometry plots of CD4+ T cells isolated from the lung at experimental day 40. **(C)** Summary data of the percentage of NP-specific cells located within each quadrant based on IFN-γ YFP and IL-5 tdTomato expression as represented in B. Graphs show mean ± SD. Data are pooled from 3-5 mice per group from two independent experiments and were analyzed by unpaired *t* test. **(D)** Schematic of the experimental timeline. **(E)** Representative flow cytometry plots of CD4+ T cells isolated from the lung at experimental day 43 and either incubated in media (unstimulated control) or stimulated with PMA/Ionomycin for 4.5 hours to assess cytokine production potential. **(F)** Summary data of the percentage of IFN-γ+ NP-specific cells and the mean fluorescence intensity of IFN-γ in NP-specific cells as represented in B. Graph shows mean ± SD. Data are pooled from 11-13 mice per group from three independent experiments and were analyzed by unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. DN, double negative. IFN-γ SP, IFN-γ YFP single positive. IL-5 SP, IL-5 tdTomato single positive. DP, double positive.

3.2.4 Diminished T-bet expression in NP-specific CD4+ T_{RM} after allergic challenge persists following heterosubtypic infection and is associated with improved disease severity

Although we observed that NP-specific CD4+ T_{RM} in the lung maintained lower T-bet expression up to 25 days following allergic challenge, it was also possible that a subsequent IAV infection could restore the T-bet levels in this population. To test this, we utilized influenza A/HKx31 H3N2 (IAV X31) for our heterosubtypic challenge as this strain contains the six internal genes of IAV PR8 including NP, therefore preserving many major CD4+ T cell epitopes while evading sterilizing immunity generated during PR8 infection [146]. Following IAV PR8 infection, mice were either left unchallenged or sensitized and challenged with HDM (**Fig. 3.5A**). At day 50 post-IAV PR8 infection, these mice were infected with IAV X31 under FTY720 (Fingolimod) treatment to determine the impact of re-infection and TCR stimulation on the phenotypic changes in NP-specific CD4+ T_{RM} observed after HDM sensitization and challenge (**Fig. 3.5A**). FTY720 promotes degradation of S1PR1 and thereby traps cells in the secondary lymphoid organs, preventing CD4+ T cell migration to the peripheral tissues [147]. We observed that FTY720 administration was sufficient to induce lymphopenia in the peripheral blood and prevent entry of NP-specific CD4+ T cells in the lungs of mice that experienced IAV X31 alone, indicating its ability to prevent the migration of cells from the secondary lymphoid organs to the lung (**Fig. 3.5B-D**). Thus, the NP-specific CD4+ T cell population in the lung analyzed at this timepoint primarily consists of cells present in the tissue prior to heterosubtypic infection and not newly recruited CD4+ T cells.

Using this experimental protocol, we observed that following IAV X31 infection, 87-96% of NP-specific CD4+ T_{RM} in the lung expressed T-bet in mice that received a prior IAV PR8 infection but were not challenged with HDM (**Fig. 3.5E, 3.5F**). However, the NP-specific CD4+ T_{RM} cell population in the lungs of mice that also experienced a respiratory HDM challenge retained a reduced frequency of T-bet+ cells and lower level of T-bet expression compared to mice that did not undergo allergic challenge even after IAV X31 infection (**Fig. 3.5E, 3.5F**).

Although the overall frequency of ST2 expression in the NP-specific T_{RM} was lower in HDM exposed mice at this timepoint, the ST2 SP NP-specific CD4+ T_{RM} population remained increased in these mice after IAV X31 infection (**Fig. 3.5E-G**). Thus, in addition to mice exhibiting a reduced frequency of T-bet+ and increased ST2+ SP NP-specific CD4+ T cells that persisted after the conclusion of challenge with HDM, this change in the NP-specific CD4+ T_{RM} population in the lung was maintained even after heterosubtypic influenza infection and perception of cognate antigen.

Although T-bet expression in CD4+ T cells is necessary for IFN-γ expression and has been associated with protection against heterosubtypic infection, T_H1 antiviral responses can also induce immunopathology and worsened disease [30, 89, 148, 149, 158]. We thus tracked weight loss daily to assess morbidity and measured viral load in the lungs 5 days following IAV X31 infection under FTY720 treatment [**Fig 3.5A**]. Mice that underwent allergic sensitization and challenge without a prior IAV PR8 infection exhibited nearly identical weight loss to mice infected with IAV X31 alone until day 5 post-infection, and possessed no significant difference in viral load in the lung at this timepoint, demonstrating that HDM exposure alone does not impact early measures of disease upon infection with IAV X31 [**Fig 3.5H, 3.5I**]. Prior infection with IAV PR8 improved morbidity as early as day 3 post-IAV X31 challenge and led to decreased viral load at day 5 post-challenge, illustrating the protective capacity of memory cells generated in response to IAV PR8 infection [**Fig. 3.5H, 3.5I**]. However, mice sensitized and challenged with HDM following IAV PR8 infection exhibited less weight loss starting at day 3 post-IAV X31 and possessed lower viral load after heterosubtypic challenge than mice exposed to IAV PR8 but not HDM [**Fig. 3.5H, 3.5I**]. Therefore, sensitization and challenge with HDM improves early disease severity and viral clearance after influenza infection by impacting the lung-resident memory compartment, and is associated with reduced T-bet expression in NP-specific CD4+ T_{RM}.

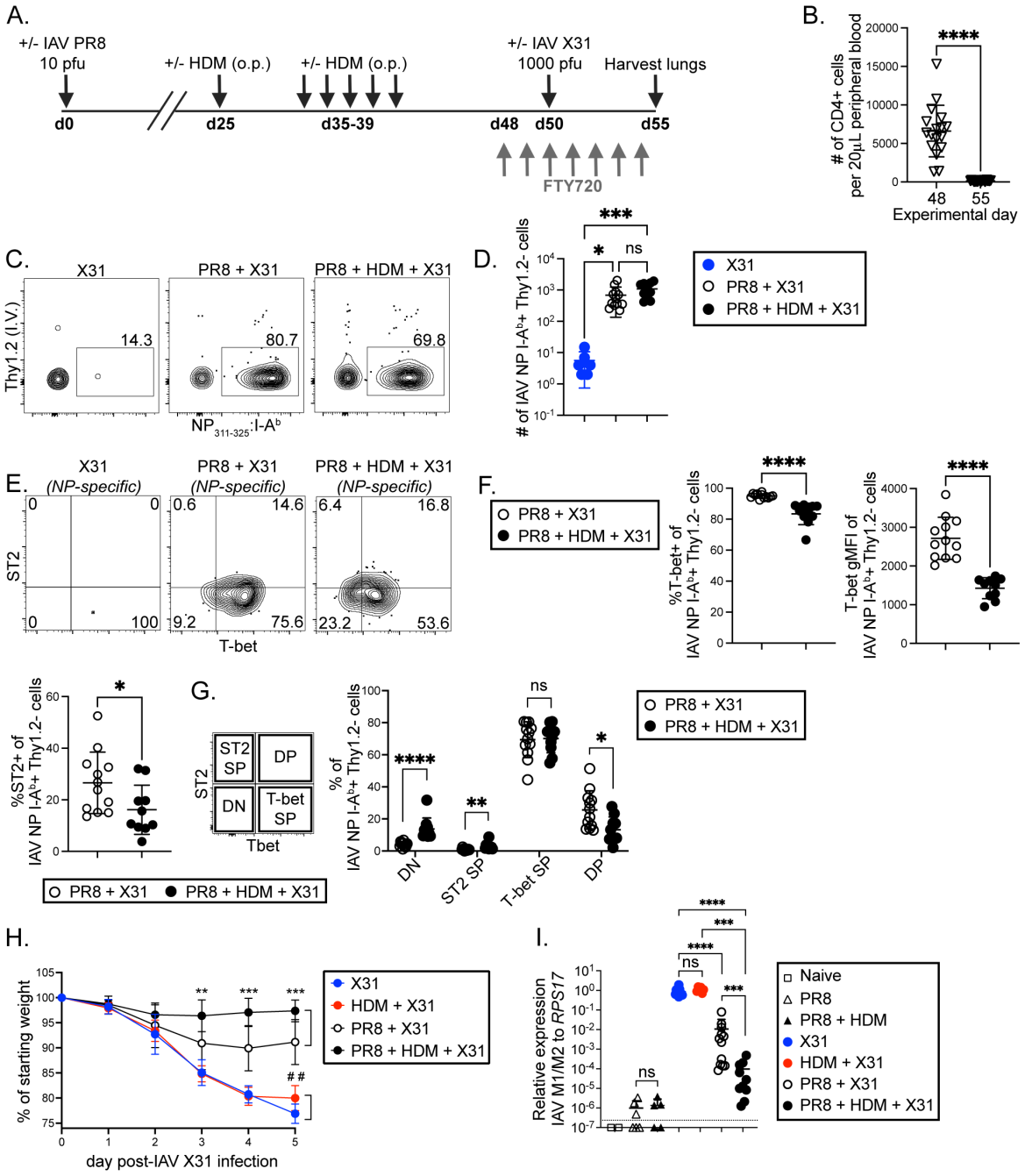

Figure 3.5. Diminished T-bet expression in NP-specific CD4+ T_{RM} after allergic challenge persists following heterosubtypic infection and is associated with improved disease severity.

(A) Schematic of the experimental timeline for data in B-I. **(B)** The total number of CD4+ cells per 20 μL peripheral blood of mice at experimental day 48 prior to FTY720 administration and at

day 55 prior to sacrifice. Graph shows mean ± SD. Data are pooled from 17-23 mice per timepoint from three independent experiments and were analyzed by unpaired *t* test. **(C)** Representative flow cytometry plots of cells isolated from the lung at experimental day 55. Box gate denotes the Thy1.2- NP-specific CD4+ cells in E. **(D)** Summary data of the number of NP-specific CD4+ cells in the lung at experimental day 55 as represented in C. Graph shows mean ± SD. Data are pooled from 6-12 mice per group from three independent experiments and were analyzed by unpaired *t* test. **(E)** Representative flow cytometry plots of NP-specific CD4+ T cells isolated from the lung at experimental day 55. **(F)** Summary data of the percentage of T-bet+ NP-specific cells, the mean fluorescence intensity of T-bet in NP-specific cells, and the percentage of ST2+ NP-specific cells as represented in E. Graphs show mean ± SD. Data are pooled from 10-12 mice per group from three independent experiments and were analyzed by unpaired *t* test. **(G)** Summary data of the percentage of NP-specific cells located within each quadrant based on ST2 and T-bet expression as represented in E. Graphs show mean ± SD. Data are pooled from 10-12 mice per group from three independent experiments and were analyzed by unpaired *t* test. **(H)** Percentage of starting (day 0 post-X31 infection) weight over time from experimental days 50-55. Asterisks (*) denote significant statistical comparisons between PR8 + X31 and PR8 + HDM + X31 groups, while octothorpes (#) signify significant statistical comparisons between X31 and HDM + X31 groups. Graphs show mean ± SD. Data are pooled from 6-12 mice per group from five independent experiments and were analyzed by unpaired *t* test. **(I)** Amount of influenza virus RNA present in lung tissue as assessed by qPCR and normalized to *Rps17* expression. Dotted line indicates limit of detection. Graphs show mean ± SD. Data are pooled from 2-12 mice per group from five independent experiments and were analyzed by Mann-Whitney test. *, $P < 0.05$; ** or ##, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. DN, double negative. ST2 SP, ST2 single positive. T-bet SP, T-bet single positive. DP, double positive.

3.2.5 Aeroallergen exposure following IAV PR8 infection induces less pro-inflammatory cytokine expression in the lungs upon heterosubtypic challenge

We previously observed that HDM exposure following IAV PR8 infection improves viral load and morbidity upon heterosubtypic challenge compared to mice previously exposed to IAV PR8 alone. A number of cytokines have been associated with increased weight loss following influenza infection, namely IL-6, TNF-α, and IFN-γ [148, 158, 159]. Thus, we assessed the expression of these cytokines in the lung tissue 5 days following heterosubtypic challenge with IAV X31 [**Fig 3.6A**]. We observed that HDM exposure alone prior to IAV X31 infection increases the level of *IFNG*, *IL6*, and *TNF* transcript compared to mice only infected with IAV X31 [**Fig 3.6B**]. However, in mice that were initially infected with IAV PR8, additional exposure to HDM reduces the level of IFNG and IL6, but not TNF, expression after IAV X31 infection, suggesting that allergic sensitization and challenge impacts the memory compartment and leads to reduced expression of these pro-inflammatory cytokines [**Fig 3.6B**]. To further understand if the decreased expression of these cytokines in the lung tissue and T-bet in NP-specific CD4+ T_{RM} was associated with a lower viral burden, we assessed the correlation between these parameters. We observed that there was a significant positive correlation between *IL6* expression, *IFNG* expression, the level of T-bet expression in NP-specific CD4+ T_{RM}, and viral load at day 5 post-IAV X31 infection [**Fig 3.6C**]. In summary, in mice previously infected with IAV PR8, additional exposure to HDM impacts the lung-resident memory compartment and leads to lower expression of pro-inflammatory cytokines and correlates with lower viral burden after heterosubtypic influenza challenge.

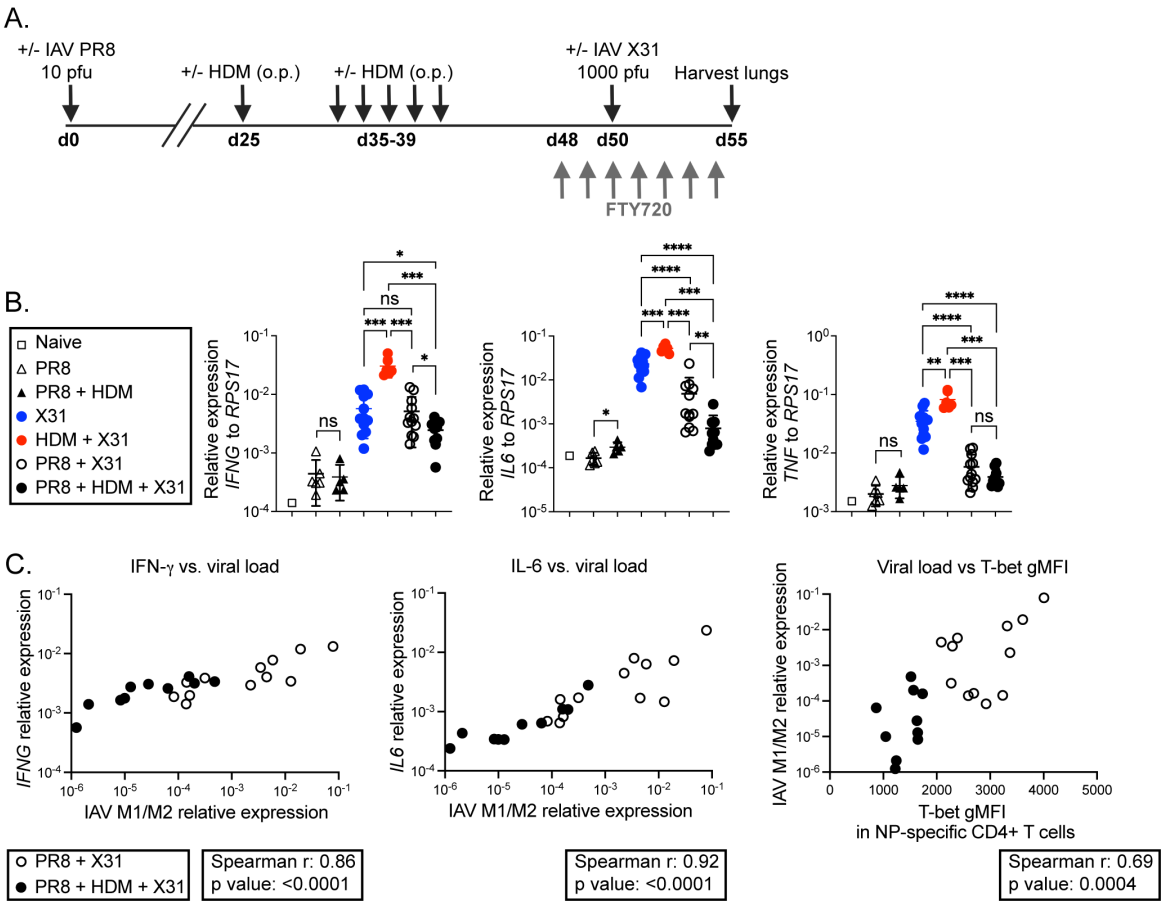

Figure 3.6. Aeroallergen exposure following IAV PR8 infection induces less pro-inflammatory cytokine expression in the lungs upon heterosubtypic challenge.

(A) Schematic of the experimental timeline for data in B-I. **(B)** Expression of *IFNG*, *IL6*, and *TNF* transcripts in lung tissue relative to *RPS17*. Graphs show mean ± SD. Data are pooled from 2-12 mice per group from five independent experiments and were analyzed by Mann-Whitney test. **(C)** Correlation between *IFNG* expression, *IL6* expression, level of T-bet expression in NP-specific CD4+ T_{RM}, and viral load. Data are pooled from 10-12 mice per group from three independent experiments and were analyzed by Spearman correlation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

3.3 Discussion

Our data demonstrate that a population of NP-specific CD4+ T_H1 T_{RM} in the lung formed following influenza infection can be altered in phenotype and function by respiratory HDM, a cysteine protease, or IL-33 exposure. The influenza-specific and HDM-specific lung-resident CD4+ T cell populations analyzed in this study are not cross-reactive and are not primed after exposure to the other immunogenic stimulus. However, the influenza-specific CD4+ T_{RM} in the lung expressed lower levels of the T_H1 transcription factor T-bet, T_H1 cytokine IFN-γ, and higher levels of the IL-33 receptor ST2 in mice that were challenged with HDM allergen following influenza infection compared to unchallenged mice without becoming T_H2-differentiated or regulatory T cells. Furthermore, the changes in T-bet expression in the lung-resident NP-specific CD4+ T_{RM} persisted for several weeks following the conclusion of allergic challenge and were retained even after heterosubtypic IAV infection, and were associated with improved disease severity and viral clearance.

Phenotypic and functional changes in the overall composition of the NP-specific CD4+ T cells in the lung reflect changes in the overall population as opposed to suggesting alterations in the expression of T-bet and ST2 at the individual cell level. For example, the loss of T-bet+ cells due to lung egress in conjunction with the entry of T-bet- cells would lead to similar conclusions as we did not observe an increase in the number of influenza-specific CD4+ T cells in the lung after challenge with HDM. Notwithstanding, we have demonstrated that the modulation of this compartment is persistent and impacts functionality. As NP-specific CD4+ T_H1 T_{RM} are important for protection against influenza infection, the decrease in the frequency of cells expressing T-bet and producing IFN-γ after HDM exposure has vital implications for how environmental antigens can impact long-term immunity [58, 89]. We show that HDM exposure after influenza infection improves morbidity, viral load, and pro-inflammatory cytokine production after heterosubtypic infection by impacting the lung-resident memory compartment. Some

studies have previously suggested that CD4+ T_H1 cells can promote pathology or worsen disease in response to heterosubtypic challenge with influenza [148, 149, 158]. Specifically, the production of IFN-γ in influenza infection promotes an antiviral response but is also associated with increased lung injury and weight loss [148]. Similarly, mice possessing IL-2-deficient memory CD4+ T cells produce less cytokines including IFN-γ and IL-12 during heterosubtypic influenza challenge and exhibit reduced morbidity and mortality [149]. Thus, dampening but not eliminating the T_H1 antiviral response by allergen exposure or other means may reduce immunopathology and lead to improved disease outcomes. Future investigation into the dependence of these changes in influenza-specific CD4+ T_{RM} populations and how to target these protective alterations should be performed.

We observed following IAV X31 infection, mice previously sensitized and challenged with HDM possessed higher levels of pro-inflammatory cytokine transcripts in the lungs than mice not exposed to HDM. This increase in pro-inflammatory cytokines in mice challenged with HDM prior to infection with IAV X31 was reversed if these mice were initially infected with IAV PR8. Since the course of acute IAV X31 infection was performed under FTY720 treatment, which prevents populations or memory and nascent immune cells from egressing from the secondary lymphoid organs into peripheral tissue, these differences could be due to responding immune populations present in the lung at the time of IAV X31 infection. In the absence of protective immune memory in the lung from IAV PR8 infection, cells that are localized to the lung tissue from HDM exposure could be activated in response to unchecked IAV X31 infection and producing more pro-inflammatory cytokines. When there are populations of immune memory cells that are sufficient to protect against heterosubtypic infection, and potentially modulated to be less immunopathologic from HDM exposure, they could be stimulated to produce less cytokines such as IL-6 and IFN-γ. However, these mechanisms are entirely speculative, and require further investigation to uncover what is driving these distinct responses

to IAV X31 infection with a prior HDM exposure when mice experienced an initial IAV PR8 infection or not.

4 Mechanisms of influenza-specific CD4+ resident memory T cell phenotype modulation

4.1 Introduction

Immune cell activation following exposure to HDM is attributed to several compounds excreted by the dust mite, including cysteine proteases that promote cleavage of epithelial cell tight junctions and stimulate the release and processing of the alarmin IL-33 [152, 153]. Along with these proteases, excrement from house dust mites contains lipopolysaccharide (LPS) which is a potent inducer of IL-12 and the IL-1 family member cytokine IL-18 [154-157]. These cytokines are implicated in promoting effector functions in memory CD4+ T cells, as IL-33R signaling can promote cytokine production by memory T_H2 CD4+ T cells in the absence of TCR engagement and IL-18Ra signaling can stimulate IFN-γ synthesis in CD4+ T cells in conjunction with IL-12 [133, 134].

4.2 Results

4.2.1 Acute respiratory challenge with papain or rIL-33 is sufficient to induce a decreased frequency of T-bet+ NP-specific CD4+ T_{RM} in the lung

As HDM contains LPS and several proteases, we sought to determine to what extent LPS or the cysteine protease papain alone could alter the functional phenotype of the NP-specific CD4+ T cell population in the lung (**Fig. 4.1A**). We found that acute challenge with LPS did not induce a statistically significant difference in either the percent of T-bet-expressing cells or ST2-expressing cells in the lung-resident NP-specific CD4+ T_{RM} population (**Fig. 4.1B, 4.1C**). However, after acute challenge with papain, the NP-specific CD4+ T_{RM} population exhibited a reduced frequency of T-bet expression and a small, but significant increase in the frequency of ST2+ cells, suggesting components of HDM extracts that induce IL-33 expression may drive the

phenotypic changes in the NP-specific CD4+ T_{RM} in the lung after allergic challenge (**Fig. 4.1B, 4.1C**). Similar to what we observed earlier with HDM, acute respiratory challenge with papain was also sufficient to induce a small, but significant increase in the frequency of ST2 SP NP-specific CD4+ T_{RM}, whereas LPS did not impact this population (**Fig. 4.1B, 4.1D**). As LPS can drive production of IL-18, we next assessed the expression of IL-18Ra on lung-resident NP-specific CD4+ T_{RM} and how it was impacted by LPS exposure [156]. After IAV PR8 infection, approximately 42% of NP-specific CD4+ T_{RM} in the lung express IL-18Ra which significantly increased after LPS but not papain exposure, suggesting further differences in phenotypic modulation by various components of common allergens (**Fig. 4.1E, 4.1F**). Of note, the IL-18Ra+ NP-specific CD4+ T_{RM} population is largely ST2- (IL-18Ra SP), while a similar proportion of ST2+ NP-specific CD4+ T_{RM} expresses ST2 alone (ST2 SP) or together with IL-18Ra (DP) (**Fig. 4.1E, 4.1G**). Together, these data suggest that while LPS exposure can alter IL-18Ra expression on NP-specific CD4+ T_{RM}, papain but not LPS exposure is sufficient to induce a decreased frequency of T-bet and increased frequency of ST2 expression in the NP-specific CD4+ T_{RM} population in the lung, recapitulating what we observed with HDM administration.

As papain and various components of HDM can induce local IL-33 and IL-18 production, and we observed that NP-specific CD4+ T_{RM} in the lung of mice can express the receptors for these cytokines, we next assessed the function of rIL-33 and rIL-18 on modulating the frequency of T-bet expression in the lung-resident NP-specific CD4+ T_{RM} population. To determine this, we administered rIL-33 or rIL-18 to the lower respiratory tract via the oropharynx beginning 25 days post-infection with IAV PR8 (**Fig. 4.1H**). Following cytokine challenge, we observed that rIL-33, but not rIL-18 was sufficient reduce the frequency and level of T-bet expression in NP-specific CD4+ T_{RM} in the lung compared to unchallenged mice (**Fig. 4.1I, 4.1J**). Although challenge with either of these cytokines was not sufficient to alter the overall frequency of ST2+ cells in this population at this timepoint, respiratory administration of rIL-33, but not rIL-18, led to an increase in the frequency of ST2 SP NP-specific CD4+ memory T cells

in the lung similar to challenge with HDM or papain (**Fig. 4.1I-K**). Taken together, these data suggest administration of rIL-33, but not rIL-18, in an acute challenge model is sufficient to drive a decreased frequency of T-bet-expressing NP-specific CD4+ T_{RM} and increased ST2 SP NP-specific CD4+ T_{RM} in the lung.

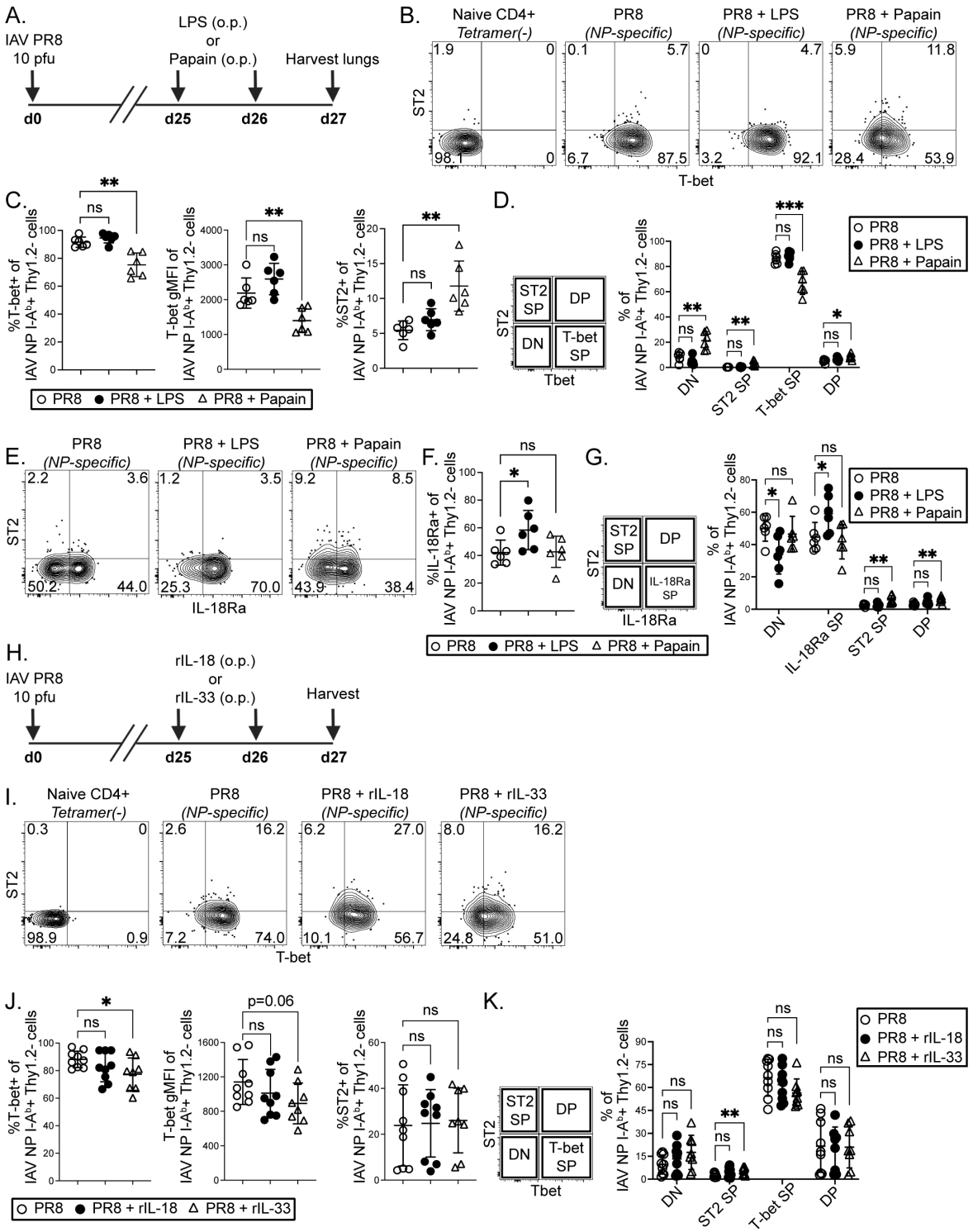

Figure 4.1. Acute respiratory challenge with papain or rIL-33 is sufficient to induce a decreased frequency of T-bet+ NP-specific CD4+ T_{RM} in the lung.

(A) Schematic of the experimental timeline for data in B-G. **(B)** Representative flow cytometry plots of CD4+ T cells isolated from the lung at experimental day 27. **(C)** Summary data of the percentage of T-bet+ NP-specific cells, the mean fluorescence intensity of T-bet in NP-specific cells, and the percentage of ST2+ NP-specific cells as represented in B. Graphs show mean ± SD. Data are pooled from 6 mice per group from two independent experiments and were analyzed by unpaired *t* test. **(D)** Summary data of the percentage of NP-specific cells located within each quadrant based on ST2 and T-bet expression as represented in B. Graphs show mean ± SD. Data are pooled from 6 mice per group from two independent experiments and were analyzed by unpaired *t* test. **(E)** Representative flow cytometry plots of CD4+ T cells isolated from the lung at experimental day 27. **(F)** Summary data of the percentage of IL-18Ra+ NP-specific cells as represented in E. Graphs show mean ± SD. Data are pooled from 6 mice per group from two independent experiments and were analyzed by unpaired *t* test. **(G)** Summary data of the percentage of NP-specific cells located within each quadrant based on ST2 and IL-18Ra expression as represented in E. Graphs show mean ± SD. Data are pooled from 6 mice per group from two independent experiments and were analyzed by unpaired *t* test. **(H)** Schematic of the experimental timeline for data in I-K. **(I)** Representative flow cytometry plots of CD4+ T cells isolated from the lung at experimental day 27. **(J)** Summary data of the percentage of T-bet+ NP-specific cells, the mean fluorescence intensity of T-bet in NP-specific cells, and the percentage of ST2+ NP-specific cells as represented in I. Graphs show mean ± SD. Data are pooled from 8-9 mice per group from three independent experiments and were analyzed by unpaired *t* test. **(K)** Summary data of the percentage of NP-specific cells located within each quadrant based on ST2 and T-bet expression as represented in I. Graphs show mean ± SD. Data are pooled from 8-9 mice per group from three independent experiments and were analyzed by unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. DN, double negative. ST2 SP, ST2 single positive. IL-18Ra SP, IL-18Ra single positive. T-bet SP, T-bet single positive. DP, double positive.

4.3 Discussion

The present findings demonstrate that rIL-33 administration is sufficient to modulate the frequency of T-bet expression in influenza-specific CD4+ T_{RM} in the lung similarly to HDM challenge. The IL-1 family member cytokines IL-18 and IL-33 have been demonstrated to directly induce effector cytokine production in T_H1 and T_H2 CD4+ T cells, respectively [133, 134]. However, only IL-33 has been shown to do so in the absence of TCR signaling in vivo [133]. Our data indicate a new role for IL-33, as only rIL-33 was able to affect the phenotype of lung-residing NP-specific CD4+ T_{RM} even though these cells express the receptors for both IL-18 and IL-33. However, whether IL-33 is being perceived directly by CD4+ T_H1 T_{RM} or directing other immune or stromal cells that then influence CD4+ T cells in the tissue remains unclear. ILC2s and adventitial stromal cells in microanatomic niches in the lung can synergistically recruit CD4+ Th2 cells to the lung via IL-33, suggesting IL-33 can impact the composition of CD4+ T cell populations in the lung independently of ST2 signaling directly modulating protein expression [83]. Additionally, ST2 can be expressed by macrophages and other myeloid cell types and perception of IL-33 by these cells can induce chemokine production and potentially T cell recruitment [150, 151]. Further work is required to determine the extent to which IL-33 signaling directly decreases T-bet and IFN-γ expression by individual influenza-specific CD4+ T_H1 T_{RM}.

5 Concluding Remarks

Although it was appreciated that CD4+ T_H2 T_{RM} could express effector cytokines in response to sensing of IL-33 prior to these studies, it was unknown if CD4+ T_H1 T_{RM} in the lung could be phenotypically or functionally altered in response to heterologous infection or antigen exposure. We have demonstrated here that influenza NP-specific CD4+ T_H1 T_{RM} which form in the lung following IAV PR8 infection exhibited decreased T-bet and increased ST2 expression following sensitization and challenge with HDM. In addition, NP-specific CD4+ T_{RM} in the lung

are not induced to express effector cytokines following HDM exposure, but their potential to produce IFN-γ upon reactivation is reduced. The changes in T-bet expression in this population persist up to four weeks following allergic challenge and after heterosubtypic infection, and are associated with improve morbidity and viral load. A reduced frequency of T-bet expression in NP-specific CD4+ TRM can be induced by acute papain or rIL-33 challenge, suggesting a role for IL-33 in driving these changes in CD4+ T_H1 T_{RM} in the lung.

While further studies should be conducted to delineate the mechanisms by which CD4+ T_H1 T_{RM} can be modulated by antigen exposure, these findings have important implications for vaccine and therapeutic design. Alterations in lung-resident CD4+ memory populations formed by infection or vaccination in response to environmental antigens leads to the possibility that studies which assess the phenotype and function of these cells acutely after they are established may not be sufficient to understand their long-term protective potential. Various infections or disease states may uniquely alter the cytokine milieu in the lung following influenza infection or vaccination, and could potentially modulate influenza-specific CD4+ T_{RM} away from their initial phenotype in ways that were not studied here. Furthermore, this work adds to an appreciable amount of evidence that suggests a heightened T_H1 response may be maladaptive and provide less protection in the context of re-infection compared to an immune response that generates a balance of immunosuppressive or tissue repair responses as well. In general, it is clear that CD4+ memory T cells in the tissue are not terminally differentiated following acute infection, which provides opportunity to further modulate their function to treat disease or enhance protection once they are formed.

6 Glossary

DCs: dendritic cells

APCs: antigen presenting cells

MHC-II: major histocompatibility complex class II

TCR: T cell receptor

T_{FH}: T follicular helper cell

T_{eff}: effector T cell

IL-2R: IL-2 receptor

T_{EM}: T effector memory cell

T_{CM}: T central memory cell

T_{RM}: tissue resident memory T cell

iBALT: inducible bronchus-associated lymphoid tissue

T_{RH}: T resident helper cells

HDM: house dust mite

NP: nucleoprotein

IAV PR8: influenza A/Puerto Rico/8/34 H1N1

IAV X31: influenza A/HKx31 H3N2

dLN: draining lymph node

FTY720: fingolimod

LPS: lipopolysaccharide

7 References

1. Janeway, C.A. & R. Medzhitov. 2002. Innate immune recognition. *Annu Rev Immunol*. 20:197-216.

2. Aderem, A. & R.J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406(6797):782-7.

3. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol*. 2(8):675-80.

4. Müller, U., U. Steinhoff, L.F. Reis, S. Hemmi, J. Pavlovic, R.M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264(5167):1918-21.

5. Opitz, B., M. Vinzing, V. van Laak, B. Schmeck, G. Heine, S. Günther, R. Preissner, H. Slevogt, P.D. N'Guessan, J. Eitel, T. Goldmann, A. Flieger, N. Suttorp, and S. Hippenstiel. 2006. Legionella pneumophila induces IFNβ in lung epithelial cells via IPS-1 and IRF3, which also control bacterial replication. 2006 *J. Biol. Chem.* 281, 36173–36179.

6. Plumlee, C.R., C. Lee, A.A. Beg, T. Decker, H.A. Shuman, and C. Schindler. 2009. Interferons direct an effective innate response to Legionella pneumophila infection. *J. Biol. Chem*. 284, 30058–30066.

7. Schiavoni, G., C. Mauri, D. Carlei, F. Belardelli, M.C. Pastoris, and E. Proietti. 2004. Type I IFN protects permissive macrophages from Legionella pneumophila infection through an IFN-γ-independent pathway. *J. Immunol.* 173, 1266–1275.

8. Ivashkiv, L.B. & L.T. Donlin. 2014. Regulation of type I interferon responses. *Nat Rev Immunol.* 14(1):36-49.

9. Montoya, M., G. Schiavoni, F. Mattei, I. Gresser, F. Belardelli, P. Borrow, and D.F. Tough. 2002. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* 99(9):3263-71.

10. Newton, K. & V.M. Dixit. 2012. Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol* 4(3):a006049.

11. Auffray, C., D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau, and F. Geissmann. 2007. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 317:666–70.

12. Nussenzweig M.C., R.M. Steinman, B. Gutchinov, and Z.A. Cohn. 1980. Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. *J Exp Med* 152:1070–84.

13. Merad M., P. Sathe, J. Helft, J. Miller, and A. Mortha. 2013. The dendritic cell lineage: Ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol*. 31, 563–604.

14. Worbs T., S.I. Hammerschmidt, and R. Förster. 2017. Dendritic cell migration in health and disease. *Nat Rev Immunol.* 17, 30–48.

15. Gerner M.Y., P. Torabi-Parizi, and R.N. Germain. 2015. Strategically localized dendritic cells promote rapid T cell responses to lymph-borne particulate antigens. *Immunity* 42, 172–185.

16. Gerner M.Y., K.A. Casey, W. Kastenmuller, and R.N. Germain. 2017. Dendritic cell and antigen dispersal landscapes regulate T cell immunity. *J Exp Med* 214, 3105–3122.

17. den Haan J.M., S.M. Lehar, and M.J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* 192:1685–96.

18. Pooley J.L., W.R. Heath, and K. Shortman. 2001. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. *J Immunol*. 166:5327–30.

19. Kitano M., C. Yamazaki, A. Takumi, T. Ikeno, H. Hemmi, N. Takahashi, K. Shimizu, S.E. Fraser, K. Hoshino, T. Kaisho, and T. Okada. 2016. Imaging of the cross-presenting dendritic cell subsets in the skin-draining lymph node. *Proc Natl Acad Sci USA*. 113, 1044–1049.

20. Baumgarth N. 2021. The Shaping of a B Cell Pool Maximally Responsive to Infections. *Annu Rev Immunol.* 39:103-129.

21. Slifka M.K., R. Antia, J.K. Whitmire, and R. Ahmed. 1998. Humoral immunity due to long-lived plasma cells. *Immunity* 8:363–72.

22. Landsverk O.J., O. Snir, R.B. Casado, L. Richter, J.E. Mold, P. Reu, et al. 2017. Antibody-secreting plasma cells persist for decades in human intestine. *J Exp Med*. 214:309–17.

23. Arroyo E.N. & M. Pepper. 2020. B cells are sufficient to prime the dominant CD4+ Tfh response to Plasmodium infection. *J Exp Med*. 217(2):e20190849.

24. Hong S., Z. Zhang, H. Liu, M. Tian, X. Zhu, Z. Zhang, W. Wang, X. Zhou, F. Zhang, Q. Ge, B. Zhu, H. Tang, Z. Hua, and B. Hou. 2018. B Cells Are the Dominant Antigen-Presenting Cells that Activate Naive CD4+ T Cells upon Immunization with a Virus-Derived Nanoparticle Antigen. *Immunity* 49(4):695-708.e4.

25. Zhou L., M.M. Chong, and D.R. Littman. 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 30:646–55.

26. Mosmann T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* 136:2348–57.

27. Kim J., A. Woods, E. Becker-Dunn, and K. Bottomly. 1985. Distinct functional phenotypes of cloned Ia-restricted helper T cells. *J Exp Med* 162:188–201.

28. Ruterbusch M., K.B. Pruner, L. Shehata, and M. Pepper. 2020. In Vivo CD4+ T Cell Differentiation and Function: Revisiting the Th1/Th2 Paradigm. *Annu Rev Immunol*. 38:705-725.

29. Van Dyken S.J., J.C. Nussbaum, J. Lee, A.B. Molofsky, H.E. Liang, J.L. Pollack, R.E. Gate, G.E. Haliburton, C.J. Ye, A. Marson, D.J. Erle, and R.M. Locksley. 2016. A tissue checkpoint regulates type 2 immunity. *Nat Immunol*. 17(12):1381-1387.

30. Szabo S.J., S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, and L.H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655–69.

31. Zheng W. & R.A. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89:587–96.

32. Ivanov I.I., B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121–33.

33. Johnston R.J., A.C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A.L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325:1006–10.

34. Nurieva R.I., Y. Chung, G.J. Martinez, X.O. Yang, S. Tanaka, T.D. Matskevitch, Y. Wang, and C. Dong. 2009. Bcl6 mediates the development of T follicular helper cells. *Science* 325:1001–5.

35. Yu D., S. Rao, L.M. Tsai, S.K. Lee, Y. He, E.L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, J.I. Ellyard, I.A. Parish, C.S. Ma, Q. Li, C.R. Parish, C.R. Mackay, and C.G. Vinuesa. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31:457–68.

36. Martins G. & K. Calame. 2008. Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu Rev Immunol.* 26:133–69.

37. Yamane H., J. Zhu, and W.E. Paul. 2005. Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment. *J Exp Med* 202(6):793-804.

38. van Panhuys N., F. Klauschen, and R.N. Germain. 2014. T-cell-receptor-dependent signal intensity dominantly controls CD4(+) T cell polarization In Vivo. *Immunity* 41(1):63-74.

39. Tubo N.J., A.J. Pagán, J.J. Taylor, R.W. Nelson, J.L. Linehan, J.M. Ertelt, E.S. Huseby, S.S. Way, and M.K. Jenkins. 2013. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell* 153(4):785-96.

40. DiToro D., C.J. Winstead, D. Pham, S. Witte, R. Andargachew, J.R. Singer, C.G. Wilson, C.L. Zindl, R.J. Luther, D.J. Silberger, B.T. Weaver, E.M. Kolawole, R.J. Martinez, H. Turner, R.D. Hatton, J.J. Moon, S.S. Way, B.D. Evavold, and C.T. Weaver. 2018. Differential IL-2 expression defines developmental fates of follicular versus nonfollicular helper T cells. *Science* 361(6407):eaao2933.

41. Snook J.P., C. Kim, and M.A. Williams. 2018. TCR signal strength controls the differentiation of CD4+ effector and memory T cells. *Sci Immunol*. 3(25):eaas9103.

42. Kotov D.I., J.S. Mitchell, T. Pengo, C. Ruedl, S.S. Way, R.A. Langlois, B.T. Fife, and M.K. Jenkins. 2019. TCR Affinity Biases Th Cell Differentiation by Regulating CD25, Eef1e1, and Gbp2. *J Immunol.* 202(9):2535-2545.

43. Nagaoka M., Y. Hatta, Y. Kawaoka, and L.P. Malherbe. 2014. Antigen signal strength during priming determines effector CD4 T cell function and antigen sensitivity during influenza virus challenge. *J Immunol*. 193(6):2812-20.

44. Johnston R.J., Y.S. Choi, J.A. Diamond, J.A. Yang, and S. Crotty. 2012. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med* 209(2):243-50.

45. Ballesteros-Tato A., B. León, B.A. Graf, A. Moquin, P.S. Adams, F.E. Lund, and T.D. Randall. 2012. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* 36(5):847-56.

46. Jones N., E.E. Vincent, J.G. Cronin, S. Panetti, M. Chambers, S.R. Holm, S.E. Owens, N.J. Francis, D.K. Finlay, and C.A. Thornton. 2019. Akt and STAT5 mediate naïve human CD4+ T-cell early metabolic response to TCR stimulation. *Nat Commun.* 10(1):2042.

47. Zhu J., J. Cote-Sierra, L. Guo, and W.E. Paul. 2003. Stat5 activation plays a critical role in Th2 differentiation. *Immunity* 19(5):739-48.

48. Cote-Sierra J., G. Foucras, L. Guo, L. Chiodetti, H.A. Young, J. Hu-Li, J. Zhu, and W.E. Paul. 2004. Interleukin 2 plays a central role in Th2 differentiation. *Proc Natl Acad Sci U S A* 101(11):3880-5.

49. Li J., E. Lu, T. Yi, and J.G. Cyster. 2016. EBI2 augments Tfh cell fate by promoting interaction with IL-2-quenching dendritic cells. *Nature* 533(7601):110-4.

50. Krishnaswamy J.K., U. Gowthaman, B. Zhang, J. Mattsson, L. Szeponik, D. Liu, R. Wu, T. White, S. Calabro, L. Xu, M.A. Collet, M. Yurieva, S. Alsén, P. Fogelstrand, A. Walter, W.R. Heath, S.N. Mueller, U. Yrlid, A. Williams, and S.C. Eisenbarth. 2017. Migratory CD11b+ conventional dendritic cells induce T follicular helper cell-dependent antibody responses. *Sci Immunol*. 2(18):eaam9169.

51. Honda T., J.G. Egen, T. Lämmermann, W. Kastenmüller, P. Torabi-Parizi, and R.N. Germain. 2014. Tuning of antigen sensitivity by T cell receptor-dependent negative feedback controls T cell effector function in inflamed tissues. *Immunity* 40(2):235-247.

52. Torabi-Parizi P., N. Vrisekoop, W. Kastenmuller, M.Y. Gerner, J.G. Egen, and R.N. Germain. 2014. Pathogen-related differences in the abundance of presented antigen are reflected in CD4+ T cell dynamic behavior and effector function in the lung. *J Immunol*. 192(4):1651-1660.

53. Pepper M. & M.K. Jenkins. 2011. Origins of CD4+ effector and central memory T cells. *Nat Immunol.* 12:467–71.

54. Sallusto F., D. Lenig, R. Förster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708–12.

55. Pepper M., A.J. Pagán, B.Z. Igyártó, J.J. Taylor, and M.K. Jenkins. 2011. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* 35(4):583-95.

56. Nguyen Q.P., T.Z. Deng, D.A. Witherden, and A.W. Goldrath. 2019. Origins of CD4+ circulating and tissue-resident memory T-cells. *Immunology* 157(1):3-12.

57. Chapman T.J. & D.J. Topham. 2010. Identification of a unique population of tissue-memory CD4+ T cells in the airways after influenza infection that is dependent on the integrin VLA-1. *J Immunol.* 184(7):3841-9.

58. Teijaro J.R., D. Turner, Q. Pham, E.J. Wherry, L. Lefrançois, and D.L. Farber. 2011. Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *J Immunol.* 187(11):5510-4.

59. Hondowicz B.D., D. An, J.M. Schenkel, K.S. Kim, H.R. Steach, A.T. Krishnamurty, G.J. Keitany, E.N. Garza, K.A. Fraser, J.J. Moon, W.A. Altemeier, D. Masopust, and M. Pepper. 2016. Interleukin-2-Dependent Allergen-Specific Tissue-Resident Memory Cells Drive Asthma. *Immunity* 44(1):155-166.

60. Beura L.K., N.J. Fares-Frederickson, E.M. Steinert, M.C. Scott, E.A. Thompson, K.A. Fraser, J.M. Schenkel, V. Vezys, and D. Masopust. 2019. CD4+ resident memory T cells dominate immunosurveillance and orchestrate local recall responses. *J Exp Med* 216(5):1214-1229.

61. Ugur M., O. Schulz, M.B. Menon, A. Krueger, and O. Pabst. 2014. Resident CD4+ T cells accumulate in lymphoid organs after prolonged antigen exposure. *Nat Commun*. 5:4821.

62. Stolley J.M., T.S. Johnston, A.G. Soerens, L.K. Beura, P.C. Rosato, V. Joag, S.P. Wijeyesinghe, R.A. Langlois, K.C. Osum, J.S. Mitchell, and D. Masopust. 2020. Retrograde migration supplies resident memory T cells to lung-draining LN after influenza infection. *J Exp Med* 217(8):e20192197.

63. Beura L.K., S. Wijeyesinghe, E.A. Thompson, M.G. Macchietto, P.C. Rosato, M.J. Pierson, J.M. Schenkel, J.S. Mitchell, V. Vezys, B.T. Fife, S. Shen, and D. Masopust. 2018. T Cells in Nonlymphoid Tissues Give Rise to Lymph-Node-Resident Memory T Cells. *Immunity* 48(2):327-338.e5.

64. Tubo N.J., B.T. Fife, A.J. Pagan, D.I. Kotov, M.F. Goldberg, and M.K. Jenkins. 2016. Most microbe-specific naïve CD4+ T cells produce memory cells during infection. *Science* 351:511–14.

65. Harrington L.E., K.M. Janowski, J.R. Oliver, A.J. Zajac, and C.T. Weaver. 2008. Memory CD4 T cells emerge from effector T-cell progenitors. *Nature* 452:356–60.

66. McKinstry K.K., T.M. Strutt, B. Bautista, W. Zhang, Y. Kuang, A.M. Cooper, and S.L. Swain. 2014. Effector CD4 T-cell transition to memory requires late cognate interactions that induce autocrine IL-2. *Nat Commun*. 5:5377.

67. Bautista B.L., P. Devarajan, K.K. McKinstry, T.M. Strutt, A.M. Vong, M.C. Jones, Y. Kuang, D. Mott, and S.L. Swain. 2016. Short-lived antigen recognition but not viral infection at a defined checkpoint programs effector CD4 T cells to become protective memory. *J Immunol.* 197(10):3936–49.

68. Marshall H.D., A. Chandele, Y.W. Jung, H. Meng, A.C. Poholek, I.A. Parish, R. Rutishauser, W. Cui, S.H. Kleinstein, J. Craft, and S.M. Kaech. 2011. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity* 35(4):633-46.

69. Löhning M., A.N. Hegazy, D.D. Pinschewer, D. Busse, K.S. Lang, T. Höfer, A. Radbruch, R.M. Zinkernagel, and H. Hengartner. 2008. Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *J Exp Med* 205(1):53-61.

70. Hondowicz, B.D., K.S. Kim, M.J. Ruterbusch, G.J. Keitany, and M. Pepper. 2018. IL-2 is required for the generation of viral-specific CD4+ Th1 tissue-resident memory cells and B cells are essential for maintenance in the lung. *Eur J Immunol*. 48:80–86.

71. Ciucci T., M.S. Vacchio, Y. Gao, F. Tomassoni Ardori, J. Candia, M. Mehta, Y. Zhao, B. Tran, M. Pepper, L. Tessarollo, D.B. McGavern, and R. Bosselut. 2019. The Emergence and Functional Fitness of Memory CD4+ T Cells Require the Transcription Factor Thpok. *Immunity* 50(1):91-105.e4.

72. Gasper D.J., M.M. Tejera, and M. Suresh. 2014. CD4 T-cell memory generation and maintenance. *Crit Rev Immunol*. 34(2):121-46.

73. Lenz D.C., S.K. Kurz, E. Lemmens, S.P. Schoenberger, J. Sprent, M.B. Oldstone, and D. Homann. 2004. IL-7 regulates basal homeostatic proliferation of antiviral CD4+T cell memory. *Proc Natl Acad Sci U S A* 101(25):9357–62.

74. Kondrack R.M., J. Harbertson, J.T. Tan, M.E. McBreen, C.D. Surh, and L.M. Bradley. 2003. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J Exp Med* 198(12):1797–806.

75. Seddon B., P. Tomlinson, and R. Zamoyska. 2003. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat Immunol*. 4(7):680–6.

76. Purton J.F., J.T. Tan, M.P. Rubinstein, D.M. Kim, J. Sprent, and C.D. Surh. 2007. Antiviral CD4+ memory T cells are IL-15 dependent. *J Exp Med* 204(4):951–61.

77. Iijima N. & A. Iwasaki. 2014. T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* 346:93–98.

78. Moyron-Quiroz, J.E., J. Rangel-Moreno, K. Kusser, L. Hartson, F. Sprague, S. Goodrich, D.L. Woodland, F.E. Lund, and T.D. Randall. 2004. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nat Med*. 10:927–934.

79. Rangel-Moreno, J., D.M. Carragher, M. de la Luz Garcia-Hernandez, J.Y. Hwang, K. Kusser, L. Hartson, J.K. Kolls, S.A. Khader, and T.D. Randall. 2011. The development of inducible bronchus-associated lymphoid tissue depends on IL-17. *Nat Immunol*. 12:639–646.

80. Veatch J.R., S.M. Lee, C. Shasha, N. Singhi, J.L. Szeto, A.S. Moshiri, T.S. Kim, K. Smythe, P. Kong, M. Fitzgibbon, B. Jesernig, S. Bhatia, S.S. Tykodi, E.T. Hall, D.R. Byrd, J.A. Thompson, V.G. Pillarisetty, T. Duhen, A. McGarry Houghton, E. Newell, R. Gottardo, and S.R. Riddell. 2022. Neoantigen-specific CD4+ T cells in human melanoma have diverse differentiation states and correlate with CD8+ T cell, macrophage, and B cell function. *Cancer Cell* 40(4):393-409.e9.

81. Hirahara K., K. Shinoda, Y. Endo, T. Ichikawa, and T. Nakayama. 2018. Maintenance of memory-type pathogenic Th2 cells in the pathophysiology of chronic airway inflammation. *Inflamm. Regen*. 38:10.

82. Dahlgren M.W. & A.B. Molofsky. 2019. Adventitial Cuffs: Regional Hubs for Tissue Immunity. *Trends Immunol*. 40(10):877-887.

83. Dahlgren M.W., S.W. Jones, K.M. Cautivo, A. Dubinin , J.F. Ortiz-Carpena, S. Farhat, K.S. Yu, K. Lee, C. Wang, A.V. Molofsky, A.D. Tward, M.F. Krummel, T. Peng, and A.B. Molofsky. 2019. Adventitial Stromal Cells Define Group 2 Innate Lymphoid Cell Tissue Niches. *Immunity* 50(3):707-722.e6.

84. Allie S.R. & T.D. Randall. 2020. Resident Memory B Cells. *Viral Immunol*. 33(4):282-293.

85. Schenkel J.M., K.A. Fraser, L.K. Beura, K.E. Pauken, V. Vezys, and D. Masopust. 2014. T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science* 346(6205):98-101.

86. Sridhar S., S. Begom, A. Bermingham, K. Hoschler, W. Adamson, W. Carman, T. Bean, W. Barclay, J.J. Deeks, and A. Lalvani. 2013. Cellular immune correlates of protection against symptomatic pandemic influenza. *Nat Med*. 19:1305–1312.

87. Wilkinson T.M., C.K. Li, C.S. Chui, A.K. Huang, M. Perkins, J.C. Liebner, R. Lambkin-Williams, A. Gilbert, J. Oxford, B. Nicholas, K.J. Staples, T. Dong, D.C. Douek, A.J. McMichael, and X.N. Xu. 2012. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. *Nat Med*. 18(2):274-80.

88. Hua L., S. Yao, D. Pham, L. Jiang, J. Wright, D. Sawant, A.L. Dent, T.J. Braciale, M.H. Kaplan, and J. Sun. 2013. Cytokine-dependent induction of CD4+ T cells with cytotoxic potential during influenza virus infection. *J. Virol*. 87:11884–11893.

89. Teijaro J.R., D. Verhoeven, C.A. Page, D. Turner, and D.L. Farber. 2010. Memory CD4 T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms. *J. Virol*. 84:9217–9226.

90. Strutt T.M., K.K. McKinstry, J.P. Dibble, C. Winchell, Y. Kuang, J.D. Curtis, G. Huston, R.W. Dutton, and S.L. Swain. 2010. Memory CD4+ T cells induce innate responses independently of pathogen. *Nat Med*. 16:558–564: 1p: 564

91. Marshall N.B., A.M. Vong, P. Devarajan, M.D. Brauner, Y. Kuang, R. Nayar, E.A. Schutten, C.H. Castonguay, L.J. Berg, S.L. Nutt, and S.L. Swain. 2017. NKG2C/E marks the unique cytotoxic CD4 T cell subset, ThCTL, generated by influenza infection. *J Immunol*. 198:1142–1155.

92. Soudja S.M., C. Chandrabos, E. Yakob, M. Veenstra, D. Palliser, and G. Lauvau. 2014. Memory-T-cell-derived interferon-γ instructs potent innate cell activation for protective immunity. *Immunity* 40:974–988.

93. Son Y.M., I.S. Cheon, Y. Wu, C. Li, Z. Wang, X. Gao, Y. Chen, Y. Takahashi, Y.-X. Fu, A.L. Dent, et al. 2021. Tissue-resident CD4+ T helper cells assist the development of protective respiratory B and CD8+ T cell memory responses. *Sci Immunol*. 6:eabb6852.

94. Swarnalekha N., D. Schreiner, L.C. Litzler, S. Iftikhar, D. Kirchmeier, M. Künzli, Y.M. Son, J. Sun, E.A. Moreira, and C.G. King. 2021. T resident helper cells promote humoral responses in the lung. *Sci Immunol*. 6:eabb6808.

95. Laidlaw B.J., N. Zhang, H.D. Marshall, M.M. Staron, T. Guan, Y. Hu, L.S. Cauley, J. Craft, and S.M. Kaech. 2014. CD4+ T cell help guides formation of CD103+ lung-resident memory CD8+ T cells during influenza viral infection. *Immunity* 41:633–645.

96. Belz G.T., D. Wodarz, G. Diaz, M.A. Nowak, and P.C. Doherty. 2002. Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice. *J. Virol*. 76:12388–12393.

97. Zhao J., J. Zhao, A.K. Mangalam, R. Channappanavar, C. Fett, D.K. Meyerholz, S. Agnihothram, R.S. Baric, C.S. David, and S. Perlman. 2016. Airway Memory CD4(+) T Cells Mediate Protective Immunity against Emerging Respiratory Coronaviruses. *Immunity* 44(6):1379-91.

98. Thomas S.Y., G.S. Whitehead, M. Takaku, J.M. Ward, X. Xu, K. Nakano, M.R. Lyons-Cohen, H. Nakano, K.M. Gowdy, P.A. Wade, and D.N. Cook. 2018. MyD88-dependent dendritic and epithelial cell crosstalk orchestrates immune responses to allergens. *Mucosal Immunol*. 11(3):796-810.

99. Zhao J., H. Shang, X. Cao, Y. Huang, X. Fang, S. Zhang, M. Xie, J. Xie, and X. Liu. 2017. Association of polymorphisms in TLR2 and TLR4 with asthma risk: An update meta-analysis. *Medicine (Baltimore)* 96(35):e7909.

100. Trompette A., S. Divanovic, A. Visintin, C. Blanchard, R.S. Hegde, R. Madan, P.S. Thorne, M. Wills-Karp, T.L. Gioannini, J.P. Weiss, and C.L. Karp. 2009. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457(7229):585-8.

101. Wan H., H.L. Winton, C. Soeller, D.C. Gruenert, P.J. Thompson, M.B. Cannell, G.A. Stewart, D.R. Garrod, and C. Robinson. 2000. Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. *Clin Exp Allergy* 30(5):685-98.

102. Jacquet A., V. Campisi, M. Szpakowska, M.E. Dumez, M. Galleni, and A. Chevigné. 2017. Profiling the Extended Cleavage Specificity of the House Dust Mite Protease Allergens Der p 1, Der p 3 and Der p 6 for the Prediction of New Cell Surface Protein Substrates. *Int J Mol Sci*. 18(7):1373.

103. Schulz O., H.F. Sewell, and F. Shakib. 1998. Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J Exp Med* 187(2):271-5.

104. Hewitt R.J. & C.M. Lloyd. 2021. Regulation of immune responses by the airway epithelial cell landscape. *Nat Rev Immunol*. 21(6):347-362.

105. Kabata H., A.L. Flamar, T. Mahlakõiv, S. Moriyama, H.R. Rodewald, S.F. Ziegler, and D. Artis. 2020. Targeted deletion of the TSLP receptor reveals cellular mechanisms that promote type 2 airway inflammation. *Mucosal Immunol*. 13(4):626-636.

106. Lai J.F., L.J. Thompson, and S.F. Ziegler. 2020. TSLP drives acute TH2-cell differentiation in lungs. *J Allergy Clin Immunol*. 146(6):1406-1418.e7.

107. Briot A., C. Deraison, M. Lacroix, C. Bonnart, A. Robin, C. Besson, P. Dubus, and A. Hovnanian. 2009. Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. *J Exp Med*. 206(5):1135-47.

108. Wilson S.R., L. Thé, L.M. Batia, K. Beattie, G.E. Katibah, S.P. McClain, M. Pellegrino, D.M. Estandian, and D.M. Bautista. 2013. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. *Cell* 155(2):285-95.

109. Ochiai S., F. Jagot, R.L. Kyle, E. Hyde, R.F. White, M. Prout, A.J. Schmidt, H. Yamane, O. Lamiable, G. Le Gros, and F. Ronchese. 2018. Thymic stromal lymphopoietin drives the development of IL-13+ Th2 cells. *Proc Natl Acad Sci U S A* 115(5):1033-1038.

110. Rochman Y., K. Dienger-Stambaugh, P.K. Richgels, I.P. Lewkowich, A.V. Kartashov, A. Barski, G.K. Khurana Hershey, W.J. Leonard, and H. Singh. 2018. TSLP signaling in CD4+ T cells programs a pathogenic T helper 2 cell state. *Sci Signal*. 11(521):eaam8858.

111. Ting H.A. & J. von Moltke. 2019. The Immune Function of Tuft Cells at Gut Mucosal Surfaces and Beyond. *J Immunol*. 202(5):1321-1329.

112. Yuan Q., N. Peng, F. Xiao, X. Shi, B. Zhu, K. Rui, J. Tian, and L. Lu. 2023. New insights into the function of Interleukin-25 in disease pathogenesis. *Biomark Res*. 11(1):36.

113. Montoro D.T., A.L. Haber, M. Biton, V. Vinarsky, B. Lin, S.E. Birket, F. Yuan, S. Chen, H.M. Leung, J. Villoria, N. Rogel, G. Burgin, A.M. Tsankov, A. Waghray, M. Slyper, J. Waldman, L. Nguyen, D. Dionne, O. Rozenblatt-Rosen, P.R.Tata, H. Mou, M. Shivaraju, H. Bihler, M.

Mense, G.J. Tearney, S.M. Rowe, J.F. Engelhardt, A. Regev, and J. Rajagopal. 2018. A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. *Nature* 560(7718):319-324.

114. Bankova L.G., D.F. Dwyer, E. Yoshimoto, S. Ualiyeva, J.W. McGinty, H. Raff, J. von Moltke, Y. Kanaoka, K. Frank Austen K, and N.A. Barrett. 2018. The cysteinyl leukotriene 3 receptor regulates expansion of IL-25-producing airway brush cells leading to type 2 inflammation. *Sci Immunol*. 3(28):eaat9453.

115. Fort M.M., J. Cheung, D. Yen, J. Li, S.M. Zurawski, S. Lo, S. Menon, T. Clifford, B. Hunte, R. Lesley, T. Muchamuel, S.D. Hurst, G. Zurawski, M.W. Leach, D.M. Gorman, and D.M. Rennick. 2001. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* 15(6):985-95.

116. Liew F.Y., J.P. Girard, and H.R. Turnquist. 2016. Interleukin-33 in health and disease. *Nat Rev Immunol*. 16(11):676-689.

117. Lüthi A.U., S.P. Cullen, E.A. McNeela, P.J. Duriez, I.S. Afonina, C. Sheridan, G. Brumatti, R.C. Taylor, K. Kersse, P. Vandenabeele, E.C. Lavelle, and S.J. Martin. 2009. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* 31(1):84-98.

118. Cayrol C., A. Duval, P. Schmitt, S. Roga, M. Camus, A. Stella, O. Burlet-Schiltz, A. Gonzalez-de-Peredo, and J.P. Girard. 2018. Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33. *Nat Immunol*. 19(4):375-385.

119. Plantinga M., M. Guilliams, M. Vanheerswynghels, K. Deswarte, F. Branco-Madeira, W. Toussaint, L. Vanhoutte, K. Neyt, N. Killeen, B. Malissen, H. Hammad, and B.N. Lambrecht. 2013. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 38(2):322-35.

120. Lewkowich I.P., S.B. Day, J.R. Ledford, P. Zhou, K. Dienger, M. Wills-Karp, and K. Page. 2011. Protease-activated receptor 2 activation of myeloid dendritic cells regulates allergic airway inflammation. *Respir Res*. 12(1):122.

121. Mikhak Z., J.P. Strassner, and A.D. Luster. 2013. Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4. *J Exp Med* 210(9):1855-69.

122. Machiels B., M. Dourcy, X. Xiao, J. Javaux, C. Mesnil, C. Sabatel, D. Desmecht, F. Lallemand, P. Martinive, H. Hammad, M. Guilliams, B. Dewals, A. Vanderplasschen, B.N. Lambrecht, F. Bureau, and L. Gillet. 2017. A gammaherpesvirus provides protection against allergic asthma by inducing the replacement of resident alveolar macrophages with regulatory monocytes. *Nat Immunol*. 18(12):1310-1320.

123. Kumamoto Y., M. Linehan, J.S. Weinstein, B.J. Laidlaw, J.E. Craft, and A. Iwasaki. 2013. CD301b^{+} dermal dendritic cells drive T helper 2 cell-mediated immunity. *Immunity* 39(4):733-43.

124. Sokol C.L., R.B. Camire, M.C. Jones, and A.D. Luster AD. 2018. The Chemokine Receptor CCR8 Promotes the Migration of Dendritic Cells into the Lymph Node Parenchyma to Initiate the Allergic Immune Response. *Immunity* 49(3):449-463.e6.

125. Gold M.J., F. Antignano, T.Y. Halim, J.A. Hirota, M.R. Blanchet, C. Zaph, F. Takei, and K.M McNagny. 2014. Group 2 innate lymphoid cells facilitate sensitization to local, but not systemic, TH2-inducing allergen exposures. *J Allergy Clin Immunol*. 133(4):1142-8.

126. Halim T.Y., C.A. Steer, L. Mathä, M.J. Gold, I. Martinez-Gonzalez, K.M. McNagny, A.N. McKenzie, and F. Takei. 2014. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40(3):425-35.

127. Halim T.Y., Y.Y. Hwang, S.T. Scanlon, H. Zaghouani, N. Garbi, P.G. Fallon, AND A.N. McKenzie. 2016. Group 2 innate lymphoid cells license dendritic cells to potentiate memory TH2 cell responses. *Nat Immunol*. 17(1):57-64.

128. Oliphant C.J., Y.Y. Hwang, J.A. Walker, M. Salimi, S.H. Wong, J.M. Brewer, A. Englezakis, J.L. Barlow, E. Hams, S.T. Scanlon, G.S. Ogg, P.G. Fallon, and A.N. McKenzie.

2014. MHCII-mediated dialog between group 2 innate lymphoid cells and CD4(+) T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity* 41(2):283-95.

129. Anderson K.G., K. Mayer-Barber, H. Sung, L. Beura, B.R. James, J.J. Taylor, L. Qunaj, T.S. Griffith, V. Vezys, D.L. Barber, and D. Masopust. 2014. Intravascular staining for discrimination of vascular and tissue leukocytes. *Nat Protoc*. 9(1):209-22.

130. Moon J.J., H.H. Chu, M. Pepper, S.J. McSorley, S.C. Jameson, R.M. Kedl, and M.K. Jenkins. 2007. Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* 27(2):203-13.

131. Legoux F.P. & J.J. Moon. 2012. Peptide:MHC tetramer-based enrichment of epitope-specific T cells. *J Vis Exp*. (68):4420.

132. Pepper M., J.L. Linehan, A.J. Pagán, T. Zell, T. Dileepan, P.P. Cleary, and M.K. Jenkins. 2010. Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. *Nat Immunol*. 11(1):83-9.

133. Guo L., Y. Huang, X. Chen, J. Hu-Li, J.F. Urban Jr, and W.E. Paul. 2015. Innate immunological function of TH2 cells in vivo. *Nat Immunol*. 16(10):1051-9.

134. Robinson D., K. Shibuya, A. Mui, F. Zonin, E. Murphy, T. Sana, S.B. Hartley, S. Menon, R. Kastelein, F. Bazan, and A. O'Garra. 1997. IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. *Immunity* 7(4):571-81.

135. Jain A., R. Song, E.K. Wakeland, and C. Pasare. 2018. T cell-intrinsic IL-1R signaling licenses effector cytokine production by memory CD4 T cells. *Nat Commun*. 9(1):3185.

136. Crowe S.R., S.C. Miller, D.M. Brown, P.S. Adams, R.W. Dutton, A.G. Harmsen, F.E. Lund, T.D. Randall, S.L. Swain, and D.L. Woodland. 2006. Uneven distribution of MHC class II epitopes within the influenza virus. *Vaccine* 24(4):457-67.

137. Cabrera-Perez J., S.A. Condotta, B.R. James, S.W. Kashem, E.L. Brincks, D. Rai, T.A. Kucaba, V.P. Badovinac, and T.S. Griffith. 2015. Alterations in antigen-specific naive CD4 T cell

precursors after sepsis impairs their responsiveness to pathogen challenge. *J Immunol*. 194(4):1609-20.

138. Meisel C., K. Bonhagen, M. Löhning, A.J. Coyle, J.C. Gutierrez-Ramos, A. Radbruch, and T. Kamradt. 2001. Regulation and function of T1/ST2 expression on CD4+ T cells: induction of type 2 cytokine production by T1/ST2 cross-linking. *J Immunol*. 166(5):3143-50.

139. Guo L., G. Wei, J. Zhu, W. Liao, W.J. Leonard, K. Zhao, and W. Paul. 2009. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proc Natl Acad Sci U S A* 106(32):13463-8.

140. Obst R., H. van Santen, D. Mathis, and C. Benoist. 2005. Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. *J Exp Med* 201(10):1555-65.

141. Geginat J., F. Sallusto, and A. Lanzavecchia. 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med* 194(12):1711-9.

142. Löhning M., A. Stroehmann, A.J. Coyle, J.L. Grogan, S. Lin, J.C. Gutierrez-Ramos, D. Levinson, A. Radbruch, and T. Kamradt. 1998. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc Natl Acad Sci U S A* 95(12):6930-5.

143. Baumann C., W.V. Bonilla, A. Fröhlich, C. Helmstetter, M. Peine, A.N. Hegazy, D.D. Pinschewer, and M. Löhning. 2015. T-bet- and STAT4-dependent IL-33 receptor expression directly promotes antiviral Th1 cell responses. *Proc Natl Acad Sci U S A* 112(13):4056-61.

144. Zhang D.H., L. Cohn, P. Ray, K. Bottomly, and A. Ray. 1997. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem*. 272(34):21597-603.

145. Finotto S., M.F. Neurath, J.N. Glickman, S. Qin, H.A. Lehr, F.H. Green, K. Ackerman, K. Haley, P.R. Galle, S.J. Szabo, J.M. Drazen, G.T. De Sanctis, and L.H. Glimcher. 2002.

Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 295(5553):336-8.

146. Rutigliano J.A., S. Sharma, M.Y. Morris, T.H. Oguin 3rd, J.L. McClaren, P.C. Doherty, and P.G. Thomas. 2014. Highly pathological influenza A virus infection is associated with augmented expression of PD-1 by functionally compromised virus-specific CD8+ T cells. *J Virol*. 88(3):1636-51.

147. Mandala S., R. Hajdu, J. Bergstrom, E. Quackenbush, J. Xie, J. Milligan, R. Thornton, G.J. Shei, D. Card, C. Keohane, M. Rosenbach, J. Hale, C.L. Lynch, K. Rupprecht, W. Parsons, and H. Rosen. 2002. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* 296(5566):346-9.

148. Schmit T., K. Guo, J.K. Tripathi, Z. Wang, B. McGregor, M. Klomp, G. Ambigapathy, R. Mathur, J. Hur, M. Pichichero, J. Kolls, and M.N. Khan. 2022. Interferon-γ promotes monocyte-mediated lung injury during influenza infection. *Cell Rep*. 38(9):110456.

149. McKinstry K.K., F. Alam, V. Flores-Malavet, M.Z. Nagy, S. Sell, A.M. Cooper, S.L. Swain, and T.M. Strutt. 2019. Memory CD4 T cell-derived IL-2 synergizes with viral infection to exacerbate lung inflammation. *PLoS Pathog*. 15(8):e1007989.

150. Joshi A.D., S.R. Oak, A.J. Hartigan, W.G. Finn, S.L. Kunkel, K.E. Duffy, A. Das, and C.M. Hogaboam. 2010. Interleukin-33 contributes to both M1 and M2 chemokine marker expression in human macrophages. *BMC Immunol*. 11:52.

151. Kurowska-Stolarska M., B. Stolarski, P. Kewin, G. Murphy, C.J. Corrigan, S. Ying, N. Pitman, A. Mirchandani, B. Rana, N. van Rooijen, M. Shepherd, C. McSharry, I.B. McInnes, D. Xu, and F.Y. Liew. 2009. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol*. 183(10):6469-77.

152. Wan H., H.L. Winton, C. Soeller, E.R. Tovey, D.C. Gruenert, P.J. Thompson, G.A. Stewart, G.W. Taylor, D.R. Garrod, M.B. Cannell, and C. Robinson. 1999. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest*. 104(1):123-33.

153. Scott I.C., J.B. Majithiya, C. Sanden, P. Thornton, P.N. Sanders, T. Moore, M. Guscott, D.J. Corkill, J.S. Erjefält, and E.S. Cohen. 2018. Interleukin-33 is activated by allergen- and necrosis-associated proteolytic activities to regulate its alarmin activity during epithelial damage. *Sci Rep*. 8(1):3363.

154. Valerio C.R., P. Murray, L.G. Arlian, and J.E. Slater. 2005. Bacterial 16S ribosomal DNA in house dust mite cultures. *J Allergy Clin Immunol*. 116(6):1296-300.

155. de Boer J.D., J.J.T.H. Roelofs, A.F. de Vos, R. de Beer, M. Schouten, T.J. Hommes, A.J. Hoogendijk, O.J. de Boer, I. Stroo, J.S. van der Zee, C. Van't Veer, and T. van der Poll. 2013. Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. *Am J Respir Cell Mol Biol*. 48(3):382-9.

156. Manigold T., U. Böcker, P. Traber, T. Dong-Si, M. Kurimoto, C. Hanck, M.V. Singer, and S. Rossol. 2000. Lipopolysaccharide/endotoxin induces IL-18 via CD14 in human peripheral blood mononuclear cells in vitro. *Cytokine* 12(12):1788-92.

157. Mantovani A., C.A. Dinarello, M. Molgora, and C. Garlanda. 2019. Interleukin-1 and Related Cytokines in the Regulation of Inflammation and Immunity. *Immunity* 50(4):778-795.

158. Califano D., Y. Furuya, S. Roberts, D. Avram, A.N.J. McKenzie, and D.W. Metzger. 2018. IFN-γ increases susceptibility to influenza A infection through suppression of group II innate lymphoid cells. *Mucosal Immunol*. 11(1):209-219.

159. Felgenhauer J.L., J.E. Brune, M.E. Long, A.M. Manicone, M.Y. Chang, T.L. Brabb, W.A. Altemeier, and C.W. Frevert. 2020. Evaluation of Nutritional Gel Supplementation in C57BL/6J Mice Infected with Mouse-Adapted Influenza A/PR/8/34 Virus. *Comp Med*. 70(6):471-486.

160. Tang X., L.S.M. Kreuk, C. Cho, R.J. Metzger, and C.D.C. Allen. 2022. Bronchus-associated macrophages efficiently capture and present soluble inhaled antigens and are capable of local Th2 cell activation. *Elife*. 11:e63296.